THE BRITISH JOURNAL OF
EXPERIMENTAL PATHOLOGY

VOL. X APRIL, 1929 No. 2

A METHOD FOR THE STUDY OF BACTERIOPHAGE MULTIPLICATION IN BROTH.

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Forwarded for publication December 21st, 1928.

THE course of bacteriophage multiplication in broth has been studied by a number of observers since d'Herelle and its general features are now well established. There is a latent period covering from 45 minutes (d'Herelle) to 5½ hours (Marshall) followed by a rapid increase that continues in logarithmic fashion for some hours. As a certain concentration is attained the curve of increase flattens out suddenly and the phage concentration remains steady or falls slightly with further incubation of the phage. These features seem to be common to all the examples that have been described, but there are great variations in the relative and absolute durations of the various phases. Doerr and Gruniger (1922) claimed that if the sensitive culture was in the logarithmic phase of its growth when the phage was added, multiplication of the phage began immediately without a lag phase. No confirmation of this finding has appeared and d'Herelle's experiments made directly to test the point never showed phage increase before 45 minutes.

Despite practical unanimity on these points there is still no generally accepted view as to their exact significance. d'Herelle interprets the course of phage multiplication by considering that each phage particle invades a sensitive bacterium, multiplies within it and at a certain stage disrupts it by internal pressure, causing simultaneously lysis of the organism and liberation into the medium of from 6 to 60 descendant particles. Of the theories opposed to the conceptions of d'Herelle several give a similar description of the course of multiplication, namely liberation of a number of active corpuscular units usually postulated as of bacterial origin, with lysis of each bacterium. The theories of Otto and Munter and of Hadley belong to this group. The two rather similar theories of Bordet and Doerr, which see the phenomena as a dysfunction of the bacterial cell by which it either gives rise to spontaneously autolysing descendants (Bordet) or liberates a stimulating growth hormone into the medium (Doerr), would, on the other hand, both seem to require that phage

increase should take place steadily rather than in sudden steps. Bail's theory that each new phage particle represents a definite unit of the chromatin hereditary substance (Erbmasse) of the bacterium lysed, postulates an increase of one phage particle with each bacterium lysed, *i.e.* a steady unitary increase.

In a recent review Bronfenbrenner (1928) expresses the view "that the phage far from being the product of lysis is regenerated during the stage of active multiplication of susceptible bacteria preceding the lysis." He bases this finding on the results of various authors with gelatine media in which multiplication of phage occurs without macroscopically visible lysis, and on the lack of exact correspondence between the times of phage increase and of visible lysis in broth cultures. His description of the process of lysis envisages two stages. In the first, the presence of phage stimulates the bacteria, activating intracellular ferments and increasing the rate of multiplication. Some of the products of this increased activity (bacteriophage) escape into the medium and in turn produce a stimulating effect on newly formed cells. The second stage, which may or may not follow the first, consists of a swelling of the bacteria on account of the increased osmotic pressure within, following intracellular digestion, and eventual rupture by the imbibition of water. This view, therefore, also implies that the first stages of bacteriophage multiplication though they may be rapid are not discontinuous.

Bronfenbrenner's description of the mechanism of the actual lysis is supported by adequate experimental evidence and is almost certainly correct, but his reasons for dissociating phage multiplication from lysis are less convincing. d'Herelle has offered what seem to be adequate explanations of the apparent differences in the times of lysis and of phage multiplication in his 1926 monograph (p. 121).

The only *direct* evidence that has been published to substantiate the theory of step-like increases is that of d'Herelle. Using a very active Shiga phage he followed the course of phage increase in a volume of 10 c.c. of broth to which he had added one or at most two phage particles. At intervals of 30 minutes 2 representative drops were spread out on agar slopes, suitably diluted if necessary to give isolated plaques. After incubation the numbers of plaques per drop at the various intervals were tabulated (Table I).

TABLE I.

	Time in minutes after addition of phage.									
	30.	60.	90.	120.	150.	180.	210.	240.	270.	300.
Plaques per drop	0	0	0	0	4	4·5	7	106	152	5000

(Table constructed from d'Herelle's figures.)

Three sudden increases in titre are evident with intermediate phases in which the phage concentration remains more or less constant. Assuming that one phage particle only was present in the original volume the three rises shown would represent the third, fourth and fifth successive liberations of a new generation of phage particles.

It is obvious that if such a step-like increase is to be observed by this

method an extraordinary uniformity must exist amongst the phage particles to ensure that each shall be adsorbed to, multiply within and lyse a sensitive organism in the same length of time. d'Herelle states that it is necessary to use a phage of absolutely maximal activity and a highly sensitive bacterial species, to start with a single phage particle and to use a large excess of bacteria. With less active phages the time taken to complete the cycle varies, perhaps widely, with each phage particle so that once multiplication begins there are liberations taking place at irregular intervals and no periodicity can be seen from mass samplings of the culture.

In view of the facts that d'Herelle's demonstration of increase by sudden steps is quite neglected by some current theories and that no independent confirmation has yet been published it does not seem redundant to describe a method by which the definitely step-like character of the first stages of phage increase can be shown for weak as well as for strong phages.

The technique is as follows:

A freshly made filtrate from a culture lysed overnight is titrated by plating successive dilutions (usually tenfold) with the sensitive strain on agar. The plates are incubated at 37° C. until the plaques are clearly visible (5–6 hours usually). From the plaque count the volume of the filtrate containing one phage particle is calculated and a suitable amount added to 10 c.c. of a growing broth culture (4 hours) of the sensitive organism to give one particle for each 0·03 c.c. of the culture. After thorough mixing volumes of 0·02 c.c. are removed in capillary pipettes previously calibrated. The pipettes are sealed at the capillary end and placed in a water-bath at 37°. At the same time an effort is made to maintain the tube containing the mixture of phage and culture at about 37° during manipulations. As soon as the required number of pipettes have been filled, 40 or 60 in most experiments, 10 lots of 0·02 c.c. are gently blown out on to an agar surface, spread over a reasonable area by tilting the plates, and dried off as quickly as possible in the incubator. At suitable intervals batches of ten sealed capillaries are opened and their contents spread similarly.

If the phage counts are satisfactory and calculation accurate, the preliminary platings will show areas of bacterial growth bearing one or no plaques, or occasionally two. In each tube, therefore, there is present none, one or at most two phage particles. Those containing none will show no evidence of phage action no matter how long they are incubated. By plating the *whole content* of numerous tubes containing one and two phage particles at various intervals, it is possible to follow accurately the first stage of phage multiplication. If a given phage multiplies according to the method envisaged by d'Herelle producing say from 20 to 30 particles with each lysis of each invaded bacterium, none of the spread areas should show plaque counts between 2 and 20. If, on the other hand, Bail's view is correct any number may be found and increase should be fairly regular.

The results obtained by the method showed at once the correctness of d'Herelle's interpretation. Two characteristic examples showing typical behaviour will be given in tabular form (Tables II and III).

This phage B has been described elsewhere (Burnet and MacKie, 1929). It is a moderately active one producing small clearly defined plaques. The table shows no counts lying between 2 and 10, and it seems most likely that in the case of this phage 10 or 12 particles result from lysis of each bacterium. Great variation in activity amongst different phage particles is shown. At 3 hours, for instance, tubes containing 1, 1 and 2 particles respectively have shown no multiplication. The count of 73 must represent a partially completed

liberation of the second generation of active particles, while in the tube giving confluent lysis many third generation particles must have been produced. The minimal time in which lysis can be completed seems to be about one hour but the numbers are not large enough to make this certain.

In the next example a bacteriophage No. 8 acting on a rough strain of *B. sanguinarium* 398R was used (Table III). This phage has been used in numerous experiments described in previous papers (Burnet, 1927, 1928).

TABLE II.—*Multiplication of Phage B on a White Staphylococcus (Strain SF).*

Time after addition of phage.	Number of plaques obtained from each specimen.									
32 minutes .	0	1	0	0	2	2	1	0	1	2
67 ,, .	0	20	1	0	0	0	1	0	0	...
97 ,, .	71	75	0	11	0	0	0	0	1	1
127 ,, .	0	0	0	0	1	45	0	0	0	0
180 ,, .	0	2	+++	0	0	0	1	1	73	...
240 ,, .	10	0	0	0	+++	39	0	38	...	...

+ + + = confluent lysis.

TABLE III.—*Multiplication of Phage 8 on* B. sanguinarium 398R.

Time after addition of phage.	Number of plaques produced.									
1 minute .	0	1	3	2	2	...	...	...	...	...
20–30 minutes .	0	0	1	0	2	0	1	0	0	2
35–40 ,, .	0	0	1	2	2	0	1	1	1	0
45–50 ,, .	3	0	0	1	2	41	1	1	...	...
55–60 ,, .	2	0	0	1	0	1	1	2	...	...
95–100 ,, .	0	35	107	1	44	79	44	88	...	...
120 ,, .	1	254[a]	67	93	86	...	...	...	...	...
130–135 ,, .	81	1	80	112	54	38	3	56	41	50
155–160 ,, .	2	30	88	44	78	(800)	14[b]	(2000)	140	175

Table III presents a number of points of interest. In the first place the number of tubes that fail to show any plaques in the earlier platings together with the presence of phage in all the later testings suggest first that the average number of phage particles added was about 2 to each capillary and secondly that with this phage an isolated particle sometimes fails to give rise to a plaque on agar though capable of multiplication in broth. This phage multiplies more regularly than the staphylococcal one used for the first experiment At about 100 minutes after mixing nearly all the phage particles have multiplied. Re-arranging the numbers of plaques obtained after multiplication has commenced in numerical order we get the series, 14, 30 [35, 38, 41, 41, 44, 44, 44, 50], 54, 56, 67 [78, 79, 80, 81, 86, 88, 93], 112, 140, 175, 254, about 800, about 2000. In this series the numbers are definitely grouped about 40 and about 80, and it is almost certain that this

grouping signifies that on an average 40 particles are produced at each first liberation. Counts around 40 represent multiplication of one phage particle, around 80 of 2 particles. The first multiplication was found 50 minutes after mixing but some particles showed no multiplication at 155 minutes. The first evidence of liberation of a second generation of phage particles occurred at 120 minutes and at 160 minutes two tubes showing roughly 800 and 2000 plaques had almost completed this second liberation.

An interesting correlation between size of plaque and activity of multiplication was obtained in this experiment. The two most aberrant figures obtained in the series are the 14 plaques produced from one tube at 160 minutes (*b*), and at the other extreme the 254 produced at 120 minutes (*a*). These plaques were distinctly smaller and larger respectively than others on the same plate. Measurements were made with low-power objective and eyepiece micrometer of 10 random plaques on these areas and on more average areas spread on the same plates. The average diameter of the 254 plaques was 0·81 mm., while another area on the same plate showed 67 plaques of average diameter 0·705 mm. The 14 plaques produced by one tube at 160 minutes averaged only 0·43 mm. in diameter as against an average of 0·65 mm. on an area showing 140 plaques on the same plate.

Phage 8 has been re-isolated from single plaques several times during the last three years and has never shown any evidence of heterogeneity. In the behaviour of two of the particles in the present experiment we have merely evidence of degrees of activity greater and less than the mean of the phage population. The first is liberated after a shorter interaction with the sensitive organism, and probably a greater number of particles, are produced at each liberation. The second, weaker, phase is re-liberated much later and fewer particles are produced. Each of the liberated particles, as judged by the size of plaque, possesses the same diminished activity of the parent particle. This is a small but significant addition to the evidence for d'Herelle's view of the genetic relationship between successive generations of phage particles. Further, if it be accepted that the phage used is a "pure line" stock, as seems undoubted, then the results show that variations of a phage population about its mean are persistent and do not immediately revert to a mean population in the next generation, *i. e.* the laws governing pure line variation as exemplified in Johannsen's experiments with beans and Agar's with Daphnia do not hold for bacteriophage in all instances.

The two experiments described above were carried out with a filtrate only a few hours old, and it was of interest to see whether the use of a phage kept for some weeks in the ice-chest would give different results. With phage 8 active on strain 398R an experiment was done parallel with that reported above using a filtrate that had remained six weeks in the ice-chest. The full protocols need not be given as of 26 tubes which were shown to contain phage, only one showed multiplication within the 110 minutes occupied by the experiment. This tube gave 22 plaques at 75 minutes. As practically all the tubes in the earlier experiment showed multiplication at 100 minutes there is evidently an increased lag when an older specimen of phage is used. The duration of the experiment was not sufficient to determine its amount.

This increased lag was not found with another phage active against the

same strain 398R. A six-weeks'-old filtrate showed first multiplication at 60 minutes in one experiment and at 90 minutes in another. A freshly prepared filtrate also showed its first increase at 90 minutes.

DISCUSSION.

The protocols given in the present paper seem to be capable of interpretation only in the sense that multiplication of phage particles and lysis are simultaneous. For a certain period only one plaque is produced by the contents of a tube; then suddenly 40 are produced without any intermediate stages. This can only mean that during the latter part of the interval before the larger number appeared the single plaque-producing entity originally present was multiplying under some spatial constraint which at a certain instant was suddenly released. Any alternative to the simple view that this release is simultaneous with the lysis of a single sensitive organism would be too strained and hypothetical to merit consideration.

The findings completely confirm d'Herelle's experiments with active Shiga phages. His work showed the increase by sudden steps quite clearly, but since it has been neither confirmed nor refuted by later authors, its importance for the interpretation of lysis has not been appreciated by the opponents of d'Herelle's theories. An examination of Tables II and III above will show that with phages of average activity such as were used, one could not hope to demonstrate any step-like increase by d'Herelle's method. The time of first liberation may differ widely for each phage particle. But by the method described any phage giving easily counted plaques on agar may be used to demonstrate the phenomenon, and it is hoped that the use of this method will allow a general agreement that in the first stages of bacteriophage action increase in the amount of phage is due to the liberation of particles by lysis of the bacteria in or on which they have been multiplying.

CONCLUSIONS.

A method is described by which the first stages of multiplication of a single phage particle in broth can be followed.

The results show that the first increase occurs suddenly. In the two cases described there is a sudden appearance of about 10 and about 40 demonstrable particles where one was present initially.

The time of first increase after addition of phage may vary widely for different types of phages and for different particles in the same filtrate.

Phage increase in broth is in the early stages at least due to the liberation at lysis of particles that have multiplied in or on a sensitive bacterium.

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ANAPHYLACTIC STUDIES WITH EXTRACTS OF HYDATID SCOLICES.

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Forwarded for publication December 31st, 1928.

IN an earlier paper from this Institute the author, with Bryce and Williams (1924), was unable, using small doses of alcoholic extract of scolices from sheep cysts, to sensitize guinea-pigs, whose plain muscle was later tested with sheep hydatid fluid or with the residue from the evaporation of alcoholic extracts of scolices suspended in saline. On the other hand, the plain muscle of guinea-pigs sensitized with thoroughly washed scolices suspended in saline gave good anaphylactic reactions when tested with sheep hydatid fluid. The results with alcoholic extract are open to objection on the ground that the dose used for sensitization (1·0 c.c.) was too small and that only traces of water-soluble substance could have been present in the residue from the evaporation of a similar amount of alcoholic extract used for testing. Further, the experiments in which suspensions of scolices were used for sensitization are not free from criticism, since despite repeated careful washing the scolices probably had adsorbed on their surface traces of sheep serum protein which, in view of the almost constant presence of host serum protein in hydatid fluid (Graetz, 1912; Kellaway, 1928), may have accounted for the positive results obtained.

Alcoholic extracts of scolices had, however, been shown by Fairley (1922) to be efficient antigens for complement-fixation, a finding which was subsequently confirmed by Patterson and Williams (1923). As far as complement-fixation is concerned there seems to be no doubt as to the solubility of helminthic antigens in alcohol. Meyer (1910–1912) used alcoholic extracts of tapeworms which had been previously extracted with acetone. These extracts, in addition to their activity in complement-fixation, could be used to sensitize guinea-pigs to the subsequent injection of watery extracts,

but did not produce anaphylactic shock when injected into sensitized animals. Fairley (1925) compared extracts in various strengths of alcohol of the fresh liver of *Planorbis exustus* infested with the cercariæ of *S. spindalis*, in regard to their power of deviating complement in the presence of antibody-containing sera. Alcoholic extracts (96·6% alcohol by volume) were found to be potent but yielded no biuret, Hopkins-Cole, ninhydrin or Millon reactions. The active factor could apparently be completely extracted with alcohol. Subsequently Turner (1926), using strong alcoholic cercarial extracts both for sensitizing guinea-pigs and for testing the isolated uterus, was unable to show that any appreciable amount of anaphylactic antigen was present in these extracts. He did not, however, use saline extracts of the original dry material for sensitizing and testing, and the amount of soluble substances in the suspensions of the residues after evaporation of alcoholic extract used for testing must have been very small. These experiments cannot therefore be regarded as excluding the presence of traces of protein, nor of an alcohol-soluble partial-antigen, similar in nature to that described by Meyer for the tapeworm.

Fairley (1927) showed that alcoholic extracts of cercariæ can cause a definite antibody response in animals as tested by complement-fixation. They therefore function as true antigens, which he regarded as non-protein in nature. He also showed that the antigenically active factor in cercariæ was not extracted by acetone but came out almost wholly in the absolute alcohol fraction, the saline extract of the residue after the two extractions possessing but little activity. His results with this helminth have since been paralleled in experiments with Miss Williams at this Institute, using the same acetone, alcohol and saline fractions from extraction of scolices, prepared by Mr. H. F. Holden, and used in anaphylactic experiments to be described in the present paper.

My recent studies with fluke extracts (1928[2]) have shown that in these there are probably at least two antigenic substances, a true water-soluble anaphylactic antigen* capable of both sensitizing and discharging sensitiveness, and an alcohol-soluble antigen capable of sensitizing but not of discharging sensitiveness. The experiments recorded in the present paper show that similar antigenic substances can be extracted from hydatid scolices.

METHODS.

Hydatid fluid and scolices were derived from sheep cysts obtained fresh from the Melbourne City abattoirs. Fluid was removed from the cysts with a hypodermic needle attached to a 10 or 20 c.c. Roux syringe, after exposing by dissection part of the adventitia and painting the exposed surface with pure phenol. Scolices were obtained from already opened sheep cysts, saline being placed in the cavity and pipetted off with Pasteur pipettes into large test-tubes. The scolices were allowed to settle and the excess of fluid poured off. They were then transferred into large watch-glasses, allowed to settle and the excess of saline above them carefully pipetted off. If they were used wet to make boiled watery extracts as in some of the earlier experiments they were washed four times with saline and separated by centrifugation. They were then ground up in an agate mortar with sterile sand.

* In the present paper by "anaphylactic antigen" is meant a substance capable not only of sensitizing guinea-pigs when injected subcutaneously but also of producing anaphylactic shock when injected intravenously into a sensitive animal or an anaphylactic reaction with desensitization of the isolated plain muscle.

Normally, however, no attempt was made to remove the last traces of host protein by repeated washing but the scolices were dried over sulphuric acid *in vacuo* for 24 or 48 hours. They were then ground up in an agate mortar. For alcoholic extracts 99·9 ± 0·1% alcohol was used and lower grades of alcohol were made up when required from this by volume. Hydatid fluid used for sensitizing was concentrated by evaporation *in vacuo* at 37° C. and for testing by pressure-dialysis against saline in pyroxylin membranes impermeable to the antigenic substances under investigation (Kellaway, Fairley and Williams, 1928). Ultra-filtration or pressure-dialysis against saline was carried out at 5° to 7° C. under a pressure of 300 to 400 mm. Hg. The membranes were made at 20° C. by the method of Adair (1925). Five layers were poured at five-minute intervals and half an hour allowed for final drying before immersion in 70% alcohol for twelve hours. These membranes were impermeable to proteins and colloidal starch solution but permitted the passage of erythro-dextrins.

Alcoholic extracts for sensitizing and testing were taken to dryness at 37° C. *in vacuo* and the residue suspended in saline. Watery saline extracts after centrifugation were also frequently concentrated *in vacuo* at 37° C. Female guinea-pigs weighing 90 to 130 gm. were used for sensitization and were allowed to remain with their mothers for at least a week after the sensitizing injection. After varying periods the animals were killed and their uteri without perfusion were tested by the method of Dale and Laidlaw. To avoid the use of rutting animals the method of Stockard and Papanicolaou (1919) was applied. The Ringer contained bicarbonate but no glucose and its pH, after adjustment by passing CO_2 through it, was maintained at a level between 7·3 and 7·4 and the partial pressure of CO_2 was kept correspondingly constant using the method described by Kellaway (1928[1]). The temperature was controlled electrically to within 1/10° at 37·5° C.

Hydatid fluid and extracts used for testing were always free from pharmacological activity in the doses used. The capacity of the bath was 50 c.c. except where a larger bath of 120 c.c. is mentioned. The Ringer in the bath was changed after each response. In all cases the plain muscle was desensitized with sheep serum before being tested with hydatid fluid or scolex extracts.

RESULTS.

1. *Experiments in which Sheep Hydatid Fluid was used for Sensitization.*

In these a high degree of sensitiveness to the parasitic antigen in hydatid fluid was obtained by using concentrated fluid for sensitization. For testing I used (1) hydatid fluid either unconcentrated or concentrated by pressure-dialysis to 1/10 of its volume; (2) saline suspensions of the residue from the evaporation of filtered extracts of dried scolices in 99·9% alcohol (1 gm. of scolices to 100 c.c. of alcohol). (The saline suspensions were made up so that 1·0 c.c. was equivalent to 5·0 c.c. of the original alcoholic extract); (3) watery scolex extract (1 c.c. of fresh scolices ground up with sterile sand and boiled for 1 hour in 100 c.c. of water, the supernatant fluid after centrifuging being evaporated to 1/10 of its bulk); (4) saline extracts of dried scolices (0·1 gm. of dried scolices shaken for half an hour in 10 c.c. of sterile saline, the undissolved matter being allowed to sediment).

In Protocol I are summarized the results of testing a single uterine horn from each of two guinea-pigs 26 days after the injection of 10 c.c. of unheated sheep hydatid fluid.

The bath had a capacity of 120 c.c. The doses of fluid and extracts used are expressed as their unconcentrated equivalents, in the order in which they were added to the bath.

These two experiments, which are typical, indicate that in neither alcoholic nor boiled watery extracts of scolices are there any appreciable amounts of substances capable of discharging the sensitiveness of the plain muscle to the helminthic anaphylactic antigen present in hydatid fluid. The stimulant effect of the watery extract in (*b*) was clearly pharmacological, and even after treatment with such large doses, the plain muscle reacts to hydatid fluid.

Other experiments in which the plain muscle was tested on the 20th and

22nd days gave similar results and more than once maximal anaphylactic reactions were first of all obtained with 0·005 c.c. of sheep serum. It was thought possible that reaction to the sheep serum protein might interfere with the subsequent demonstration of feeble sensitiveness to scolex extracts. Hydatid fluid heated for 1 hour at 100° C. was therefore used in some experiments for sensitization, for, as I have shown (1928[1]), the plain muscle of animals sensitized with heated hydatid fluid is not sensitive to host serum proteins. In one such experiment in which the plain muscle was tested on the 23rd day no response was obtained with 1·0 c.c. of sheep serum or with 5·0 c.c. of alcoholic extract but 2·0 c.c. of hydatid fluid gave an almost maximal response.

PROTOCOL I.—*Reactions of One Horn of the Uterus of Each of Two Guinea-Pigs Sensitized 26 Days Earlier by the Injection of 10 c.c. of Unheated Hydatid Fluid.*

	Doses for testing in the order in which they were used.	Result.
(*a*)	0·5 c.c. sheep serum	Maximal response.
	0·5 c.c. „ „	Small „
	0·5 c.c. „ „	*Nil.*
	15·0 c.c. alcoholic extract of scolices .	„
	0·01 mgm. histamine	Maximal response.
	10·0 c.c. watery extract of scolices . .	Very small „
	1·0 c.c. hydatid fluid	½ maximal „
	1·0 c.c. „ „	*Nil.*
(*b*)	0·5 c.c. sheep serum	Maximal response.
	0·5 c.c. „ „	Very small „
	20·0 c.c. watery extract	Large „
	20·0 c.c. „ „	„ „
	10·0 c.c. „ „	Smaller „
	1·0 c.c. hydatid fluid	Maximal „
	1·0 c.c. „ „	*Nil.*

The results obtained with the plain muscle of guinea-pigs sensitized with hydatid fluid and tested with unboiled saline extracts of scolices indicate that such extracts either contain a substance capable of eliciting an anaphylactic response in plain muscle sensitive to hydatid fluid, or that the sensitive plain muscle is more susceptible than the uteri of normal guinea-pigs to stimulant substances present in these extracts which were freshly made up and not dialysed. Protocol II is an example of this kind of action. It will be observed that despite the response to saline scolex extract a maximal anaphylactic response was subsequently elicited with hydatid fluid.

It appears from these experiments that the parasitic anaphylactic antigen present in hydatid fluid is not present in either boiled watery extracts or alcoholic extracts of scolices. Absence of desensitization following reactions with fresh saline extracts of scolices suggests its absence in these also and possibly the results given in Protocol II are due to the presence of suspended scolices in the hydatid fluid used for sensitization which was not filtered in every instance.

PROTOCOL II.—*Reactions of One Horn of the Uterus of a Guinea-Pig Weighing 215 gm. which had been Sensitized 23 Days Before by the Subcutaneous Injection of 15 c.c. of Sheep Hydatid Fluid.*

Doses for testing in the order in which they were used.	Result.
0·1 c.c. sheep serum	Maximal response.
0·1 c.c. „ „	*Nil.*
1·0 c.c. fresh saline extract of scolices	Maximal response.
1·0 c.c. „ „	*Nil.*
0·5 c.c. sheep hydatid fluid	Maximal response.
0·5 c.c. „ „	*Nil.*
0·002 mgm. histamine	Maximal response.

2. *Experiments in which watery extracts of scolices were used for sensitization.*

The extract used for sensitization was made by grinding up 1·0 c.c. of four times washed scolices with sterile sand and boiling for 1 hour with 150 c.c. of water. The extract was allowed to stand at 5° C. overnight and the supernatant fluid taken to dryness *in vacuo* at 37° C. The residue from evaporation of this fluid was suspended in 20 c.c. of sterile saline. This was used in a dose of 3·0 c.c. to sensitize guinea-pigs which were tested 21 days later. The animals did not acquire any sensitiveness to sheep serum since boiling had destroyed any sheep serum protein present, but became sensitive to hydatid fluid and to boiled watery extract of scolices as Protocol III shows.

PROTOCOL III.—*Reactions of the Two Horns of the Uterus of a Guinea-Pig Sensitized 21 Days Earlier by the Injection of Watery Extract of Scolices. Ringer Bath, 120 c.c.*

Testing doses in the order in which they were applied.			Result.
Left horn	5·0	c.c. hydatid fluid	Maximal response.
	5·0	c.c. „ „	*Nil.*
	0·01	mgm. histamine	Maximal response.
Right horn	0·5	c.c. sheep serum	*Nil.*
	2·0	c.c. hydatid fluid	⅓ maximal response.
	2·0	c.c. „ „	*Nil.*
	0·3	c.c. watery extract	Maximal response.
	0·3	c.c. „ „	*Nil.*

In experiments of this kind in which boiled watery extracts of scolices are used both for sensitization and testing it is possible that new antigenic substances are formed by the process of heating either from the sheep serum protein absorbed on the scolices or from some substance in the parasite (*cf.* Furth, 1926). This explanation might be offered to account for the different results obtained in experiments in which unheated saline extracts of scolices or scolices suspended in saline were used for sensitization. In these, sensitiveness to host serum protein was invariable but, in contrast to the regularity of the acquirement of sensitiveness when boiled extracts are used, sensitiveness to hydatid fluid and to unheated saline extract of scolices was irregular and, on the whole, rather late in appearance.

The plain muscle of guinea-pigs sensitized with unheated saline extracts of scolices tested on the 22nd day reacted maximally with doses of 0·0005 c.c. to 0·01 c.c. of sheep serum in a bath of 50 c.c. but failed to react to unheated saline extract of scolices or to hydatid fluid. On the other hand, in parallel experiments, the plain muscle of animals sensitized with boiled saline extracts of scolices, tested on the 23rd day was insensitive to sheep serum protein, but gave maximal reactions with 1·0 c.c. of boiled saline scolex extract dialysed to remove any pharmacologically active substance present. Fig. 1 is an example taken from a second series of similar experiments. It shows the reactions obtained on the 22nd day with the uterus of a guinea-pig sensitized with heated saline scolex extract. The plain muscle is insensitive to sheep serum but sensitive to hydatid fluid obtained from another host and to unheated saline extract of scolices. In the corresponding experiments in which unheated saline scolex extract was used for sensitization, sensitiveness to sheep serum was invariable and in experiments on the 28th day the plain muscle also reacted with unheated saline extract of scolices and with hydatid fluid.

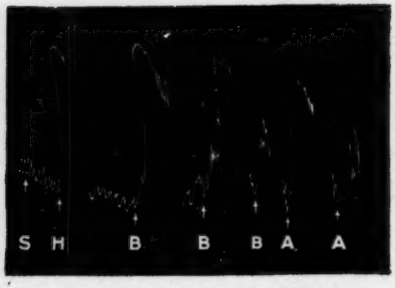

FIG. 1.—Response of one horn of the uterus of a guinea-pig weighing 290 gm. sensitized by the injection 22 days before of 2·5 c.c. of unfiltered extract of scolices (0·1 gm. of dried scolices in 10 c.c. of saline heated in a water-bath at 100° C. for half an hour). At S 0·2 c.c. of sheep serum, at H 0·002 mgm. histamine, at B 2 c.c. of hydatid fluid from a kidney cyst in a pig, and at A 1·0 c.c. of fresh saline extract of scolices added to the bath (50 c.c.)

Protocol IV gives two examples of the kind of results obtained.

PROTOCOL IV.—*Reactions of One Horn of the Uterus of Two Guinea-Pigs Sensitized 28 Days Previously with 2·5 c.c. of Unheated Saline Scolex Extract (0·1 gm. Dried Scolices in 10 c.c. Saline.)*

	Testing doses in the order in which they were applied.	Result.
(*a*)	0·1 c.c. sheep serum	Maximal response.
	4·0 c.c. alcoholic extract of scolices (residue from the evaporation of 4 c.c. of extract—0·4 gm. in 100 c.c. 99·9% alcohol	*Nil.*
	0·5 c.c. pig hydatid fluid	„
	1·0 c.c. fresh saline extract of scolices	Maximal response.
	1·0 c.c. „ „ „	Delayed stepped response.
	1·0 c.c. „ „ „	*Nil.*
(*b*)	1·0 c.c. pig hydatid fluid	Maximal response.
	1·0 c.c. „ „	*Nil.*
	0·1 c.c. sheep serum	Maximal response.
	0·1 c.c. „ „	*Nil.*
	1·0 c.c. fresh saline extract of scolices	„
	0·002 mgm. histamine	Maximal response.

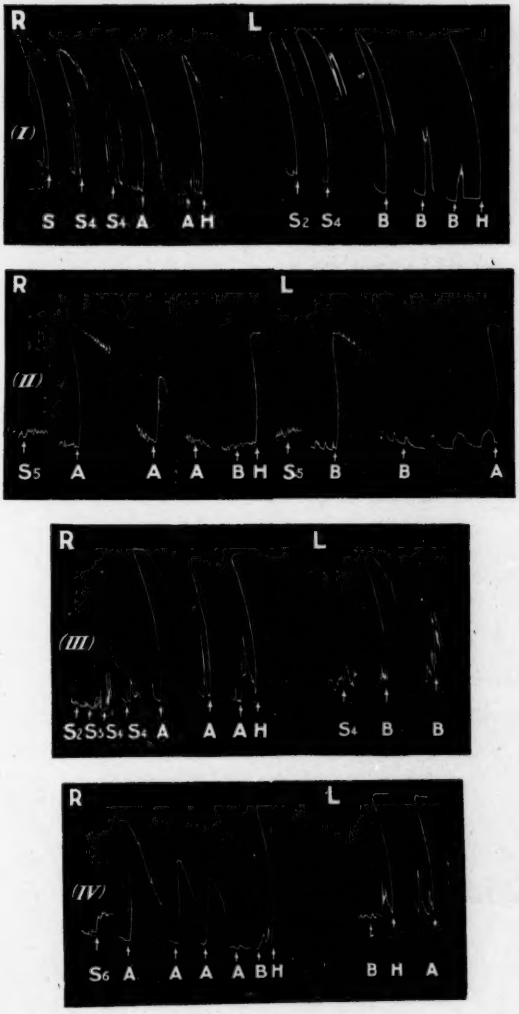

FIG. 2.—Responses of the right (R) and left (L) horns of the uteri of guinea-pigs weighing from 170 to 250 gm. 30 to 31 days after sensitization with the residue from the evaporation of 8·0 c.c. of extracts of scolices in (i) 40%, (ii) 60%, (iii) 80%, and (iv) 100% alcohol (1 gm. of dried scolices in 100 c.c.) At S 0·0075 c.c., S_2 0·001 c.c., S_3 0·01 c.c, S_4 0·4 c.c., S_5 0·5 c.c., and S_6 1·0 c.c of sheep serum. At A, 1·0 c.c. of saline extract of scolices (0·5 gm. of dried scolices to 40 c.c. of saline); at B, 1·0 c.c. of sheep hydatid fluid, and at H, 0·0001 mgm. of histamine added to the bath.

The plain muscle of animals sensitized with scolices (1·0 c.c. suspended in saline) tested on the 22nd day was insensitive or almost so to hydatid fluid (giving only a trivial response with 3·0 c.m.) and to dialysed watery extract of scolices, but reacted maximally with small doses of sheep serum. Other animals tested on the 27th day were sensitive to sheep serum, the plain muscle reacting maximally with doses of less than 0·01 c.c., and to 1·0 c.c. of fresh unboiled saline extract (0·5 gm. scolices in 40 c.c. of saline). The uteri of animals sensitized with saline scolex extract and tested on the 38th day were sensitive to sheep serum and gave nearly maximal reactions to 0·5 c.c. of saline scolex extract and to 1·0 c.c. of hydatid fluid. Hydatid fluid could be used to desensitize to saline scolex extract so that after eliciting a response to 1·0 c.c. of hydatid fluid and failing to elicit a response with a second dose, 0·5 c.c. of saline scolex extract caused no reaction. The apparently anomalous result shown in Protocol IV may be an example of this frequently observed phenomenon. It may possibly be related to the difficulty of completely removing hydatid fluid adsorbed on the surface of the scolices, though these, in all the later experiments, were very carefully washed with many changes of saline. As however, this proceeding failed to remove host protein, it is reasonable to conclude that it also failed to remove all the adsorbed protein from hydatid fluid.

3. *Experiments in which Alcoholic Extracts of Scolices were used for Sensitization.*

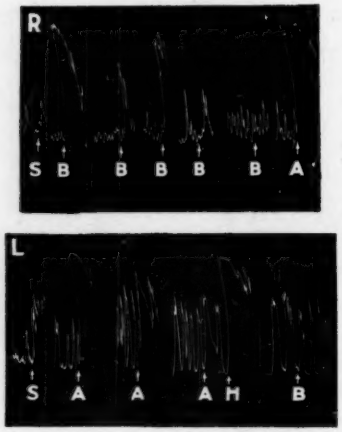

FIG. 3.—Response of the right (R) and left (L) horns of the uterus of a guinea-pig sensitized by the injection 31 days before of the residue from the evaporation of 8·0 c.c. of an extract of scolices in 80% alcohol. At S, 0·5 c.c. of sheep serum, at B, 1·0 c.c. of (pig kidney) hydatid fluid, at A, 1·0 c.c. of fresh saline extract of scolices, and at H, 0·002 mgm. of histamine added to the bath.

In Protocol V are summarized the results of a series of experiments in which guinea-pigs sensitized with the residue from the evaporation of 8·0 c.c. of alcoholic extracts in 40%, 60%, 80% and 99·9% alcohol were tested on the 30th and 31st days with sheep serum, saline scolex extracts and hydatid fluid. Fig. 2 gives the actual record in 4 of these experiments.

The results in these experiments which were carried out on two animals in each group were strikingly uniform. It will be seen that host serum protein is not taken up except in the 40% alcohol, though occasionally I have obtained evidence of sensitiveness after injecting the residue from 25 c.c. of filtered extracts of dried scolices in 100% alcohol even when the utmost care was taken (using Whatman No. 30 filter-papers) to avoid the possibility of leakage through a defective paper. With 80% and 60% alcoholic extracts sensitiveness was demonstrated both to hydatid fluid and to saline scolex extracts and whereas it was possible to desensitize with saline scolex extract to hydatid fluid (possibly because of the presence

of hydatid fluid adsorbed on the scolices used for making the extract) it was not possible to desensitize with hydatid fluid and scolex extract (Fig. 3). The 100 % extract produced no sensitiveness to hydatid fluid.

These results must be interpreted in the light of observations on guinea-pigs sensitized with saline extracts of scolices and tested with alcoholic extracts. In these experiments I have never succeeded in eliciting a response with the residue from the evaporation of an alcoholic extract nor have I been able to desensitize sensitive plain muscle to the subsequent addition of saline scolex extract. It appears then that we are concerned here with at least one "partial antigen" soluble in alcohol and capable of causing sensitization but not of making sensitive plain muscle contract nor of desensitizing it. If we assume that an anaphylactic antigen from hydatid fluid is adsorbed on the scolices, and is soluble in 40 %, 60 % and 80 % alcohol but insoluble in 99·9 % alcohol, it is only necessary to postulate one "partial antigen" to explain the results recorded above. Alternatively there may be two "partial antigens" one of which is insoluble in absolute alcohol. The former hypothesis affords the best explanation of the observed facts.

A further experiment on the solubility of hydatid antigens was carried out in co-operation with Mr. H. F. Holden in which washed, dried and powdered scolices were extracted first with acetone, then with alcohol and finally with saline. The extraction was carried out in a manner closely similar to that adopted by Fairley (1925) in his experiments with cercariæ. Similar results to those obtained by him in the bilharzia experiments have been obtained by Fairley and Miss Williams (unpublished) using these extracts in complement-fixation tests with antibody-containing sera.

The extraction was carried out as follows: Two samples each of 0·5 gm. of dry powdered scolices from sheep hydatid cysts were placed in 50 c.c. of acetone, which had been purified by fractional distillation, dried over calcium chloride and again fractionally distilled, the fraction distilling between 56° and 56·5° C. being collected. The scolices were repeatedly shaken and allowed to remain in this acetone for 24 hours at 30° C. The acetone was then replaced by fresh solvent and the scolices again extracted for 24 hours. Four such extractions were made and the acetone from these was pooled, distilled off *in vacuo* and the residue taken up in 10 c.c. of saline = *Fraction I.*

The residue of powdered scolices remaining after the acetone extraction was now dried in air at 10° C. and was extracted four times with 50 c.c. of absolute alcohol for 24 hours at 37° to 40° C. The alcohol from these extractions was pooled, distilled *in vacuo* and the residue from its evaporation taken up in 10 c.c. of saline = *Fraction II.*

The residue of scolices after the alcohol extraction weighed 0·4 gm and was suspended in 20 c.c. of physiological saline = *Fraction III.*

Guinea-pigs were sensitized by the subcutaneous injection of portions of each of these fractions and their plain muscle was tested on the 30th day with the results summarized in Protocol VI.

The experiments in which Fraction III was used for sensitization were unsatisfactory. The plain muscle of one animal sensitized with an amount equivalent to 0·2 gm. of scolices was tested on the 24th day and was extremely sensitive to sheep serum protein. After desensitization it was not possible to test the plain muscle further as it had too great a rhythm. Instead, therefore, of desensitizing the plain muscle in the bath, an attempt was made to desensitize it *in vivo* by injecting 0·2 c.c. of sheep serum subcutaneously in two other animals 24 hours before they were killed and their uteri tested.

PROTOCOL V.—*Summary of Experiments in which Alcoholic Extracts of Scolices were Used for Sensitization.*

Strength of alcohol.	Sheep serum.		Saline scolex extract.		Hydatid fluid.	
	Dose.	Result.	Dose.	Result.	Dose.	Result.
100%	1·0 c.c.	*Nil.*	1·0 c.c.	Maximal response	1·0 c.c.	*Nil.* (After treatment with several doses, still reacts to saline scolex.)
80%	0·4 to 0·7 c.c.	,,	1·0 c.c.	Maximal response. (Desensitized thereby to hydatid fluid)	1·0 c.c.	Maximal response. (After treatment with several doses, still reacts to saline scolex.)
60%	0·5 c.c.	,,	0·2 c.c.	Maximal response. (Repeated doses of 1·0 c.c. desensitize to hydatid fluid)	0·5 c.c.	Maximal response. (After treatment with repeated doses of 1·0 c.c., still reacts to saline scolex.)
40%	0·00025 c.c.	Maximal response.	1·0 c.c.	Maximal response	1·0 c.c.	Maximal response.

PROTOCOL VI.—*Results of Experiments in which* (1) *the Acetone Soluble,* (2) *the Acetone Insoluble, Alcohol Soluble Fractions of Scolices were Used for Sensitization.*

Sensitizing dose.	Sheep serum.		Saline extract of scolices.		Hydatid fluid.	
	Testing dose.	Result.	Testing dose.	Result.	Testing dose.	Result.
Acetone soluble Fraction I.—Equivalent to soluble material from 0·25 gm. scolices	0·1 c.c.	*Nil.*	1·0 c.c.	*Nil.*	1·0 c.c.	Maximal response. (2 out of 3 experiments.)
Acetone insoluble, alcohol soluble Fraction II.—Equivalent to 0·2 gm. of scolices	0·1 c.c.	Maximal response.	1·0 to 2·0 c.c.	Maximal response. (Desensitizes to hydatid fluid.)	1·0 to 2·0 c.c.	Maximal response. (Does not desensitize to scolex extract.)

The plain muscle was now insensitive to sheep serum and reacted with fresh saline scolex extract but after desensitization with this failed to react with hydatid fluid. The second horn after failing to respond to 0·1 c.c. of sheep serum gave a maximal response with 0·5 c.c. of hydatid fluid.

These results are in conformity with the experiments recorded here with alcoholic extracts of scolices and demonstrate that the "partial antigen" in scolices which sensitizes to saline scolex extracts is insoluble in acetone. The antigenic substance in scolices which causes sensitiveness to hydatid fluid, whatever its nature, appears to be soluble, to some extent at least, in acetone. It is interesting to note that *extraction of a helminth with pure dry acetone and subsequently with 99·9% alcohol does not exclude the presence of host protein in amounts detectable by the anaphylactic experiment.*

SUMMARY AND CONCLUSIONS.

The general result of these experiments is to bring the hydatid scolex into line with other helminths, the tape-worm (Meyer) and the fluke (Kellaway). The substances in scolex which are insoluble in acetone but soluble in absolute alcohol and which act as "partial antigens," causing sensitiveness but being unable to discharge it, may possibly owe their activity *in vivo* to union with some body protein in the guinea-pig producing a foreign complex to which antibody can be produced. There is, however, in addition a water-soluble substance present in saline extracts of scolices which can function as an anaphylactic antigen. Owing to the difficulty of freeing scolices from the protein substances in hydatid fluid it is not certain that this substance is not derived from hydatid fluid. Finally host (sheep) serum protein is present in extracts of scolices and extraction with pure dry acetone and with absolute alcohol does not guarantee its absence from the resulting extracts.

It is a pleasure to acknowledge my indebtedness to Dr. N. H. Fairley, who collected the materials used in these experiments from the City abattoirs and to Miss F. E. Williams who prepared dry powdered scolices for use in these experiments.

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ULTRA-FILTRATION METHODS AND THEIR APPLICATION IN BACTERIOLOGICAL AND PATHOLOGICAL STUDIES.

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Received for publication February 2nd, 1929.

ULTRA-FILTRATION, filtration through gel membranes as the filter septum, provides one of the few direct methods for investigating disperse systems. A graded series of filter membranes of progressively finer permeability, such as may be prepared under appropriate conditions from collodion, enables the separation of more and more finely dispersed fractions, and furthermore permits the order of size of the suspended particles to be ascertained.

A problem of outstanding importance in pathological science to-day consists in elucidating the nature of the so-called "filterable viruses," the causative agents of numerous diseases in man and animals. A concise survey of the essential features of this problem has been given by Barnard (1926) and reference to his paper will obviate the necessity for elaboration here. It suffices to state that these viruses have been found by bacteriologists to pass through the "filter candles" (made of porcelain or diatomaceous earth) which are known to retain efficiently ordinary bacteria (hence the name "filter passer" or "filterable virus"); and therefore, it is concluded, are of a lesser order of size than the latter. Assuming, as there is good reason to do, that these viruses are particulate, ultra-filtration obviously provides a direct method for investigating them. Naturally in considering the application of ultra-filtration methods the efficacy of the filter membrane is at once questioned. "What will a given membrane perform in way of filtration?" "What are its filtration capabilities?" "Will it retain ordinary bacteria, for example, under certain particular or under all filtration conditions?" With the view to answering such questions as these the present work was initiated, a study being made of the filtration capacities of graded ultra-filter membranes for typical bacteria. Organisms have been selected that might well serve as controls in subsequent filtration experiments.

Numerous instances of the use of ultra-filtration methods in bacteriological investigations occur in the literature, but definite information in regard to the fundamental processes involved is lacking. That membranes can be prepared to retain bacteria is, of course, well-known; for instance, Ficker (1917) has separated the toxin from *Bacillus œdematis maligni* by ultra-filtration, while Eichoff (1921) and also Meyeringh (1922) have filtered various types of organisms through Zsigmondy-Bachmann membranes of graded porosity and have demonstrated the filtration efficiency of the latter. In the present work special study has been made of the various factors operative in such filtration

experiments with the view to establishing the essential points of technique, the full appreciation of which is the key to successful application of ultra-filtration methods.

Membranes prepared from acetic acid collodion have been used, filter paper being impregnated with solutions of various concentrations and subsequently immersed in water in order to "gel" the collodion through replacement of the solvent (Bechhold, 1907). The filtration capabilities of these membranes have been studied for two reasons. First the alternative gel membranes prepared from ether-alcohol collodion are generally much less permeable than the acetic collodion ones; in fact it is not readily possible to obtain a membrane of this class that is permeable to bacteriophage which is of an order of size well below that of the filterable viruses.* On the other hand, the glacial acetic collodion membranes provide a wide range in permeability from the order of 1μ downwards. Furthermore, and secondly, the technique of preparing the acetic collodion membranes is relatively simple, gradation in permeability being attained by varying the concentration of the nitro cotton in the collodion. It becomes a matter of importance, therefore, that the practical utility of these membranes in such problems as those here considered should be systematically investigated.

ACETIC ACID COLLODION MEMBRANES.

The essential points in membrane-preparation technique have been dealt with in detail by the author (1928). The nitro-cotton used in the present work was a pyroxylin containing 10·96% nitrogen, for the analysis of which the author is indebted to Mr. C. W. Frost of the R.N. Cordite Factory, Horton Heath. Glacial acetic acid collodions varying in nitro-cotton concentration from 1·0%–6·0% by weight were prepared, the solutions being shaken mechanically until homogeneous and stored in darkness.

The viscosity of a 2·0% solution, prepared in a glass tube 9 in. long by 1 in. diameter and shaken uniformly at 25·0° C. for 6 hours, was 2·002 C.G.S. units at 25·0° C. and age 1 hour. This viscosity value, determined with a standardized viscometer of the Ostwald type, is cited for purposes of future comparison with collodions from different samples of nitro-cotton.

Method of Preparation of Membranes.

Discs of filter-paper (Schleicher and Schüll No. 575) 4 cm. in diameter, with tab to facilitate handling, were impregnated by the vacuum impregnation method. After impregnation the discs are drawn horizontally between two gold-plated rollers which are fixed at a definite distance, 0·020 cm. apart by means of accurately turned collars attached to the lower roller. [*Cf.* Procedure of Bechhold (1907) who allows discs to drain while being constantly rotated.]

* Since writing this paper the contribution of McBain and Kistler (1928) has come to hand, in which the use of cellophane as basis for ultra-filter membranes is recommended. The potentialities of this material swollen in water and alcohol/water mixtures were investigated quite independently by the author at the commencement of the present work. Results were obtained which are in full accord with the findings of McBain and Kistler, for while it was found possible to prepare membranes permeable to sugar solutions, albumin in aqueous solution was completely retained. These membranes were therefore placed in the same category as the true gel membranes from ether alcohol collodion as being insufficiently permeable for filterable virus studies.

The gold-plated nickel rollers 1 in. in diameter are mounted on a nickel-plated stand. (*Note.*—Nickel rollers not permissible since nickel is slowly attacked by acetic acid.) The rollers are clamped in a fixed position by means of the side screws. It was originally intended to allow the rollers to rotate, but owing to the high viscosity of the collodion a certain variable amount adheres to the rollers after the membrane has passed and so the present procedure was adopted. This modified technique in preparation of impregnated membranes has resulted in uniformity of membrane thickness throughout the complete range of nitro-cellulose concentration used and has thereby introduced a degree of consistency hitherto unattainable, yet so essential for comparative filtration purposes.

Sterilization of Membranes.

The usual methods employed for obtaining sterile membranes have been either to heat the membrane in water until the latter boils and allow to cool slowly, or to subject the membrane immersed in water to autoclaving at 105–110° C. Now such treatment is not without a modifying effect upon the membrane which becomes less permeable to water to the extent of 20–30%, while at higher autoclaving temperatures the membrane is rendered altogether useless. In any case the gradation in membrane permeability so carefully attained in the preparation technique becomes modified, as must inevitably result from any procedure involving heat treatment.

In the present study use has been made of the bactericidal properties of ultra-violet light. The treatment of the discs subsequent to the impregnating process is conducted in the sterile atmosphere of an ultra-violet light chamber designed by Mr. Barnard, F.R.S. The chamber is so arranged that light from the mercury vapour lamp falls directly upon all the interior surfaces, and the ventilating air enters as the warm air in the neighbourhood of the lamp ascends and passes out on that side of the chamber where the experimenter works. This device enables work to be conducted in a sterile atmosphere, sterilized rubber gloves being worn by the operator, who is able to view his manipulations through a blue glass window.

The membranes are washed in sterile distilled water for 10–14 days, transferences to fresh sterile water being made twice daily in the ultra-violet light chamber.

Careful experiments were made to determine whether or not ultra-violet light affected the membranes to modify their permeabilities. Membranes immersed in water were exposed to the direct rays from the mercury vapour lamp for various intervals of time up to one hour, at a distance of one foot from the lamp, this being the minimum distance from the light source at which work was ever likely to be conducted. In no instance was any modification of the membrane detected in studying, before and after exposure, the rate of flow of water through the membrane under standard conditions. As the total direct exposure of any membrane in actual practice is easily less than 5 minutes, it may be confidently stated that the procedure is thoroughly sound. Scores of experiments have been carried out with membranes prepared in the way described without any evidence of contamination having arisen, thus testifying to the highly satisfactory technique pursued.

Apparatus.

The form of ultra-filter used is illustrated in Fig. 1. Constituted of brass and heavily nickel-plated it has a capacity of approximately 25 c.c., taking a

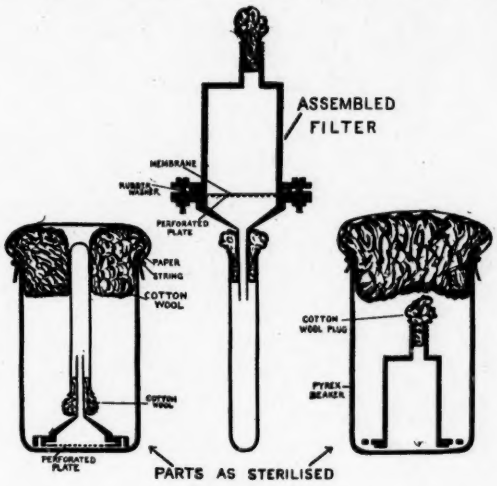


FIG. 1.

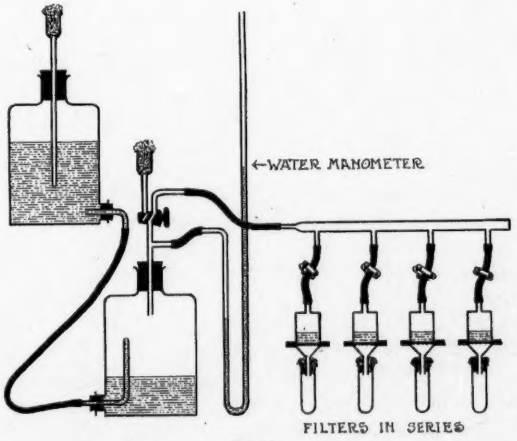


FIG. 1a.

membrane 4 cm. in diameter. The components are arranged, as shown in the figure, to be sterilized in a hot-air oven at 150° C. for 1 hour. The red rubber washer, contained in a petri-dish lined with filter-paper, is autoclaved. The assembling of the filter is carried out in the ultra-violet light chamber.

Fig. 1A shows the experimental arrangement of the apparatus for low

pressure filtration 0–80 cm. water. The source of pressure is the head of water supplied by the positions of the aspirator bottles and is used in conjunction with the water-manometer, but for higher pressures a mercury-manometer is used and pressure derived from a nitrogen cylinder *viâ* suitable reducing valve. Four filtration experiments may be conducted simultaneously under identical conditions. This is essential when running control experiments.

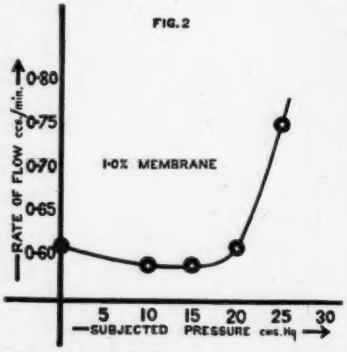
General Properties of Membranes.

In the case of the particular nitro-cotton used in this investigation a 1·0% solution in glacial acetic acid was the lowest limit of concentration which would yield a consistent membrane. Weaker solutions gave membranes the permeability of which, as indicated by the rate of flow of water, was very erratic. Increasing the concentration progressively from 1·0% the membranes became less permeable and at the same time stronger.

The standard of the membranes prepared in the manner already described was in each case uniform, the rate of flow of water (proportional to the fourth power of the radius of pores) under specified conditions invariably agreeing to within 10%, which, when one considers the nature of the membranes and the dependence of uniformity upon that of the filter-paper, may be regarded as very satisfactory. The interesting fact has been noted that membranes from any particular nitro-cellulose solution become more permeable as the solution "ages." Thus the "ageing" effects of nitro-cotton solutions manifested in lowering of viscosity are reflected too in modification of nature of the gel.

Membranes prepared from the more dilute collodions below 3·0% need to be handled with extreme care, since the gel film is very fragile. This makes it essential to know the maximum pressure for each grade of membrane that may be employed in filtration experiments in order that erroneous results may be avoided. This limit has been determined by subjecting each membrane supported on a perforated plate as in actual filtration experiment, to gradually increasing air-pressures, and after each increment measuring the rate of flow of water under standard conditions. Fig. 2 gives the curve for 1·0% membrane. The rate of flow decreases slightly and then attains a steady value, which is probably due to the fact that the membrane is being pressed more closely against the plate, the actual filtering area thereby becoming more strictly that of the holes of the plate. The permeability increases abruptly at a fairly definite pressure, which may be taken as proportional to the strength of the membrane. Having conducted such experiments for each grade of membrane the maximum filtration pressure for filtration experiments may be deduced. Thus, in the present instance the determinations were:

FIG. 2



1·0% membrane. Maximum filtration pressure = 15 cm. mercury.
1·5% „ „ „ „ = 60 „ „
2·0% „ „ „ „ = 150 „ „

For other membranes the values were well outside the upper limits of pressure being used in these experiments.

Permeability of Membranes.

The legitimacy of applying Poiseuille's law (a relationship derived theoretically and found experimentally to hold for the flow of liquids through long solid capillary tubes) in deriving permeability relationships for these membranes was investigated. The generally accepted conception of the functioning of ultra-filter membranes is that they behave in a manner analogous to a system of fine parallel capillaries orientated at right angles to the membrane surface, and therefore having length corresponding to the thickness of the membrane. Several investigators have substantiated this view in case of ether alcohol collodion membranes, by finding that Poiseuille's law does govern the flow of liquids through these (Duclaux and Errera, 1924; Bigelow and Gemberling, 1907). However, in view of certain obvious points of dissimilarity between ether alcohol collodion membranes and those being used here, it was not considered justifiable to assume the applicability of the law without obtaining some evidence therefor. Apart from the disturbing effect that must be exercised by presence of the interlacing mat of cellulose fibres, there is a definite point of difference in the processes of formation. The membrane film from glacial acetic collodion is produced by the interdiffusion of water in the collodion, the nitro-cotton solvent, acetic acid, being replaced by the non-solvent water, resulting in the coagulation of nitro-cotton. Whether complete coagulation in forms of fibrous elements, or partial coagulation—" gelling "—occurs is entirely a question of the relative bulks and distribution of the reactants, water, acetic acid and solvated nitro-cellulose aggregates. In the case of ether-alcohol collodion membranes, on the other hand, the film is allowed to gelate (*i. e.* the nitro-cellulose aggregates assume a more or less definite mutual orientation) before the solvent is washed out with water, which process only fixes the structure already potentially developed. Herein lies the reason for the wonderful uniformity of gel films that can be prepared from ether alcohol collodion.

Poiseuille's law is expressed in the following equation:

$$Q = \frac{P \pi r^4}{8 \eta 1}$$

Q = vol. liquid passing in c.c./sec.
P = pressure producing flow-dynes/sq. cm.
r = rad. of capillary cm.
η = viscosity of liquid at temp. obtaining, C.G.S. units.
1 = length of capillary, cm.

The rates of flow of water through membranes of various grades and the influence of pressure were studied. Over a range of low pressures, 10 cm. to

100 cm. water, the rate of flow was found to vary directly as the pressure, but as the pressures were further increased a gradual departure from the linear proportionality occurred, the rate of flow increasing at a slightly accelerated rate and not retaining the strictly uniform acceleration as when direct proportionality obtained. This effect may be attributed to the membrane becoming slightly distended under pressure. For standard conditions within this low-pressure range the rate of flow of water was found to vary inversely with the thickness of the membrane and by conducting experiments at various temperatures from 15·0° C.–30·0° C., the rate of flow varied inversely with the viscosity of water.

Adapted for a membrane containing n pores per unit area Poiseulle's relationship becomes—

$$Q=\frac{n P \pi r^4}{8 \eta l} \quad . \quad . \quad . \quad . \quad . \quad (1)$$

To demonstrate conclusively the proportionality between rate of flow and the fourth power of the pore radius is extremely difficult, since it is necessary to know the number as well as the size of pores in several membranes of the series. Assuming the total volumes of the pores equal to the water-content of the membrane it may be shown that the number of pores varies inversely as the square of the pore radius. Therefore the rate of flow should be directly proportional to the square of the radius throughout the series, for comparable conditions. That this is approximately the case was found by using values of r estimated by filtering suspensions of known particle size. However, there was evidence that definite departure from the law possibly occurred in the case of membranes from the more dilute solutions (1·0% and 1·5%), and this is very probable in view of certain supplementary structural evidence obtained. Nevertheless the sum total of evidence was considered to indicate that the law might justifiably be used in calculating values of pore size from the data furnished by rate of flow of water measurements, provided the pressure comes within the stipulated range 10–100 cm. water.

From equation (1) it follows that

$$r = 2l\sqrt{\frac{2 Q \eta}{P V}} \quad . \quad . \quad . \quad . \quad . \quad (2)$$

where $V = n \pi r^2 l$ = total volume of pores.

By determining the water-content of the membrane, assumed to be equal to the total volume of the pores, V may be evaluated. Then Q and l having been measured the value of r can be calculated (Hitchcock, 1926; Bjerrum and Manegold, 1927).

An alternative consists in assuming:

$$V=\frac{(100 - x)}{100} v.$$

Where x = % nitro-cellulose in gel assumed equivalent to that in collodion; *i. e.* no appreciable contraction taking place on replacement of acetic acid by water; and v = volume of gel in membrane.

It follows then that for membranes of equal thickness and determination of Q under standard conditions—

$$r = K\sqrt{\frac{Q}{(100 - x)}} \quad . \quad . \quad . \quad . \quad . \quad . \quad (3)$$

This may be shown equivalent to the relationship used by Bechhold (1908).

Membrane.	D_0.	D_1.	D_2.	Remarks.
1·0%	0·75μ	0·39μ	0·51μ	D_0 estimated from filtration *B. prodigiosus.*
1·5%	...	0·23μ	0·32μ	...
2·0%	0·20μ	0·20μ	0·25μ	D_0 estimated from filtration colloidal carbon.
2·5%	...	0·18μ	0·22μ	...
3·0%	...	0·16μ	0·18μ	...
4·0%	...	0·12μ	0·14μ	...
5·0%	0·05μ	0·077μ	0·092μ	D_0 estimated from filtration of colloidal gold.
6·0%	...	0·061μ	0·070μ	...

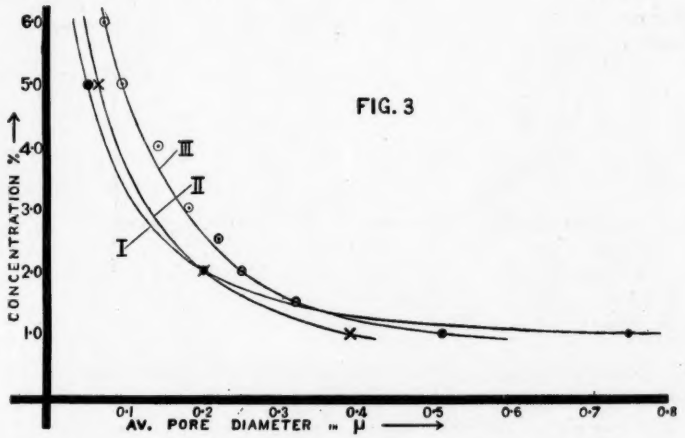


FIG. 3

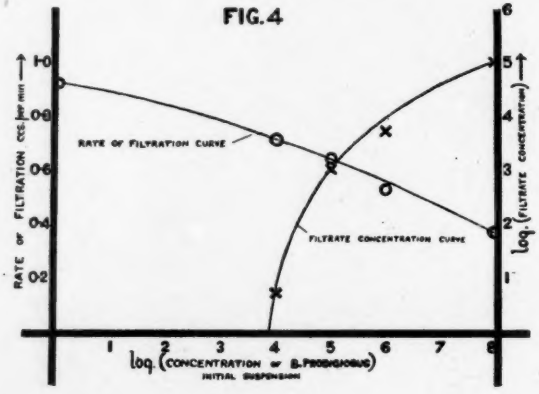
Now column D_0 of the accompanying table contains values of pore diameter deduced from filtration experiments, and column D_2 the values calculated after determining the water-content of the various membranes. Plotting these two sets of values against the nitro-cotton content of the collodion the Curves I and III respectively in Fig. 3 result. The value $d = 0{\cdot}2\,\mu$ for the 2% membrane given by the filtration of colloidal carbon is seen to be in good agreement with the value $d = 0{\cdot}25\,\mu$ given by the other method. The former value was therefore used in evaluating the constant K in equation (3) above, and thence used as the basis for calculating the pore sizes of other membranes of the series. These values are contained in column D_1 and represented in Curve II of graph Fig. 3.

FILTRATION RESULTS.

Suspensions of the following organisms have been filtered under comparable conditions through membranes of the series described, employing applied pressures ranging from 5 cm. water to 100 cm. mercury: *B. coli*, *B. prodigiosus*, *B. bronchisepticus* and bovine pleuro-pneumonia. In addition suspensions of red blood-corpuscles (horse and frog) and also bacteriophage (coli) have been examined in an analogous manner, and in each instance the influences of conditions of filtration upon filtrability has been systematically studied.

It is imperative in all comparative filtration work that the experimental conditions are consistent. For instance, to consider a few determining factors —difference in applied pressure may well give an entirely different filtration result; variation in protein-content of fluid being filtered produces variation in filtrability, especially in rate of filtration; and again the pH value

FIG. 4



of the solution determines the physical properties (viscosity, solvation, adsorbability, etc.) of the protein constituents. The precise nature of the effects of these physical factors will not be discussed here since for the purpose of the immediate problem it suffices if their influences are kept constant throughout the series of experiments. Thus in all cases solutions of comparable protein-content have been filtered and the pH values maintained strictly within the limits 7·4–7·6, while an accurate record of the applied filtration pressures has been kept.

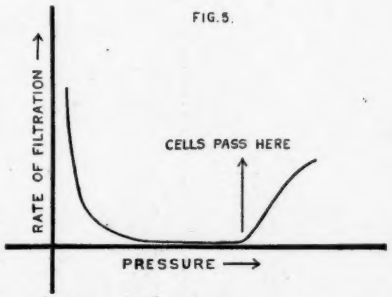
Other conditions being standardized the concentration of dispersed bacteria can influence the filtration. This is well illustrated by results obtained with *B. prodigiosus* (see Fig. 4). The more concentrated the suspension the greater the chance of an organism finding itself at the entrance of a pore in the early stages of the filtration, and passing through before protein constituents have appreciably narrowed the diameter of the pores. Naturally, when the pore size is several times that of the particle being filtered, the increase in concentration of the disperse phase will result in increase in filtration up to a certain point, beyond which further increase in concentration will result in mutual

interference at the entrance to the pore resulting in mechanical blockage. Excessive applied pressure can have the same effect. Indeed, increase in applied pressure often defeats its purpose. Especially is this so in fractional filtration, where low pressures are best, since with progressively higher pressure the more completely do the particles of the more coarsely dispersed fraction, which is to be retained, block the pores, thereby preventing the passage of the finely dispersed fraction wanted in the filtrate. The difficulty of mechanical blockage can only be satisfactorily overcome by having an arrangement for periodically reversing the pressure to clear the pores. An intermittent filter designed by Mr. J. E. Barnard, F.R.S., is being tried out in this laboratory and promises to be of distinct value in fractional filtration of such systems as extracts of minced tissues, etc., where the separation of a fine colloidally dispersed constituent from the relatively coarse suspensoidal extract of tissue *débris* has to be effected.

Red Corpuscles.

(a) *Horse Blood-Corpuscles.*—The corpuscles used were from freshly defibrinated horse blood, carefully washed in isotonic saline with aid of the centrifuge until washings no longer showed turbidity on boiling. The washed cells were suspended in a medium containing serum broth (1 vol. horse serum, 5 vols. broth) and saline (0·85 % NaCl) in the ratio 1–10 by volume. Counts were made using a Zeiss hæmocytometer and a suspension containing 100,000 cells per c.mm. was used in all experiments. This suspension was filtered through progressively less permeable membranes commencing with 1·0%. The filtrates were in all cases subjected to careful microscopic examination. The results are presented in Table I. It will be observed that membranes below 2·0% permitted the cells to pass, but that in each case the membrane effectively withheld the corpuscles until a certain pressure value was attained. This corresponds to the pressure necessary to deform the cells in forcing them through the pores of the membrane. This was conclusively demonstrated by observing the rate of filtration during the experiment. Commencing at low water pressures the filtration proceeded at quite a fair rate which, however, as the pressure was gradually increased, fell off and became dead slow corresponding without doubt to the blockage of the pores by corpuscles. Then at a definite pressure and coinciding with the appearance of red cells in the filtrate, the rate suddenly increased. A typical form of the Rate/Pressure graph is shown in Fig. 5.

FIG. 5.



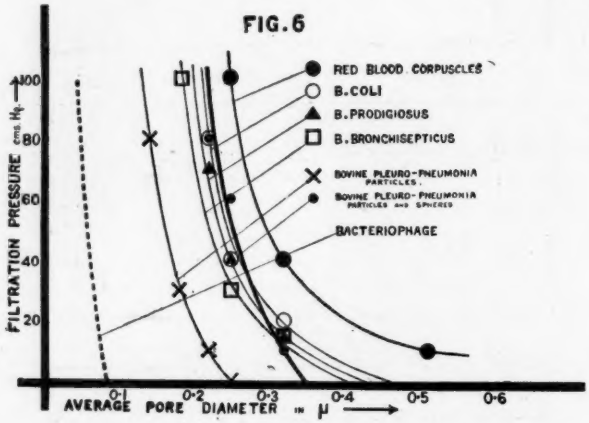
(b) *Frog Blood-Corpuscles.* (*Rana Esculenta*).—The blood was transferred immediately to isotonic citrate solution to prevent clotting and then washed as before, using citrate solution instead of saline in all but the final washings. A suspension was made in the same medium as used for horse blood-corpuscles

and analogous experiments conducted. The number of cells per c.mm. in all experiments was 12,000.

All the membranes completely retained these cells, which readily passed through Schleicher and Schüll No. 575 filter-paper, at pressures less than 5 cm. water as supplied by the depth of suspension fluid. The average dimensions of the frog blood-corpuscles which appeared ovoid in shape were determined microscopically to be length 20–22μ and width 14–16μ, while in the case of the horse blood-cells the average diameter was 5·5–6·0μ. Then, too, the frog corpuscles are nucleated which fact undoubtedly contributes to their being less readily deformed.

These two sets of experiments illustrate well the influence of applied pressure upon the filtrability of deformable cells. The pressures at which the corpuscles of horse blood pass membranes are plotted against the average pore

FIG. 6



size in Fig. 6. The cells in the filtrates with 1·5% and 2·0% membranes exhibited pronounced crenation and also some hæmolysis. The latter effect was unquestionably demonstrated by filtering suspensions of corpuscles in saline. Examination of the filtrates at various pressure intervals showed that they contained increasing amounts of heat coagulable protein and also hæmoglobin. This may have been the result of ultra-filtration of cell-contents through the distended cell-wall membrane, or what certainly happens in the case of filtration through 2·0% membranes the cell-wall becomes irreversibly distended and in some cases ruptured. This conclusion is supported by the fact that the crenation effects in the case of 1·5% filtrate invariably disappeared with time, the cells regaining their normal configuration, but in the case of 2·0% filtrates the crenation, which was very marked, appeared to be largely irreversible. Thus, although corpuscles pass a 2·0% membrane, the pressure required has an injurious effect on the cells. Deformation through pores less than one-fifth their own diameter results in irreversible distention of the cell membrane accompanied by varying degrees of hæmolysis.

B. coli.

A broth culture of the organism incubated at 37·5° C. for 12 hours and filtered through a sterile filter-paper to remove any coarse clusters, was used in these experiments. To 5 c.c. of this culture 1 c.c. of horse serum was added and the whole was diluted with saline and made comparable with the

TABLE I.

RED BLOOD CORPUSCLES (HORSE).

Pressure	cms. WATER				cms. MERCURY												
Membrane	10	20	30	40	5	10	15	20	25	30	35	40	50	60	70	80	100
1·0%	−	−	−	−	−	±	+										
1·5%	−	−	−	−	−	−	−	−	−	−	−	+	+	+			
2·0%	−	−		−	−		−					−		−		−	±
2·5%							−					−		−		−	−
3·0%												−		−		−	−

TABLE II.

B. COLI.

Pressure	cms. WATER				cms. MERCURY												
Membrane	10	20	30	40	5	10	15	20	25	30	35	40	50	60	70	80	100
1·0%	+	+		+	+	+											
1·5%	−	−		−	−	−	−	±	+	+		+		+			
2·0%								−		−	−	±	+	+		+	+
2·5%								−				−		−	−	±	+
3·0%												−		−		−	−
4·0%												−		−		−	−

TABLE III.

B. PRODIGIOSUS.

Pressure	cms. WATER				cms. MERCURY												
Membrane	10	20	30	40	5	10	15	20	25	30	35	40	50	60	70	80	100
1·0%	+	+	+	+	+	+	+										
1·5%	−	−	−	−	−	−	±	+	+	+		+		+			
2·0%	−	−		−	−	−		−	−	−	−	+		+		+	+
2·5%						−		−		−		−		−	±	+	+
3·0%									−			−		−		−	−
4·0%								−				−		−		−	−

suspensions used in other experiments. The concentration of *B. coli* was invariably of the order 100 millions per c.c. The filtration results are contained in Table II. The filtrates were in each case examined by preparing agar plate cultures and making counts under known conditions. Parallel cultures with various dilutions of the original suspension enabled the concentration of the filtrate to be expressed in terms of that of the original fluid.

Pressure is a determining factor in the filterability of *B. coli*, a certain value specific to each membrane for given conditions being necessary for the

organism to pass. The use of pressures exceeding this minimal filtration value results in higher concentration of *coli* in the filtrate, but the pressure increment necessary to effect an additional increase in relative concentration

TABLE IV

B. BRONCHISEPTICUS.

Pressure	cms. water				cms. mercury											
Membrane	10	20	30	40	5	10	15	20	25	30	35	40	50	60	70	80	100
1·0%	±	+		±	±	+											
1·5%	−	−		−	−	−	±	+	+	±		±	+				
2·0%						−		−	−	±	+	±	+			+	+
2·5%								−		−		−	−	±		±	+
3·0%								−				−		−	−	−	±
4·0%								−				−		−		−	−

TABLE V

BOVINE PLEURO-PNEUMONIA.

Pressure	cms. water				cms. mercury											
Membrane	10	20	30	40	5	10	15	20	25	30	35	40	50	60	70	80	100
1·0%	+⊕	+⊕	+⊕	+⊕	+⊕	+⊕											
1·5%	+⊖	+⊖	+⊖	+⊖	+⊖	+⊖	+⊕	+⊕	+⊕			+⊕		+⊕			
2·0%	−	+⊖	+⊖			+⊖		+⊖	+⊖			+⊖		+⊖	+⊕	+⊕	+⊕
2·5%	−	−	−	−	−	+⊖	+⊖	+⊖		+⊖		+⊖		+⊖		+⊕	+⊕
3·0%						−	−	−	−	+⊖		+⊖		+⊖		+⊖	+⊖
4·0%						−		−		−		−	−	−	−	+⊖	+⊖
5·0%												−		−		−	−

TABLE VI.

BACTERIOPHAGE (COLI).

Pressure	cms. water				cms. mercury											
Membrane	10	20	30	40	5	10	15	20	25	30	35	40	50	60	70	80	100
1·0%	+	+		+													
1·5%	+	+			+												
2·0%	+		+			+											
2·5%	+	+				+											
3·0%	+	+		+		+											
4·0%	+	+				+		+									
5·0%	+	+			+			+						+			
6·0%												+		+			+

KEY TO TABLES

+ = ORGANISM INVARIABLY PASSES
− = ORGANISM INVARIABLY RETAINED
± = BORDERLINE PRESSURE ; SOMETIMES + SOMETIMES −
⊕ = SPHERES PASS
⊖ = SPHERES RETAINED

APPROXIMATE FILTRATE CONCENTRATIONS ARE INDICATED BY FIGURES EXPRESSING DILUTION OF ORIGINAL, e.g. $\times 10^3$ = 1/1000 ORIGINAL.

of filtrate increases rapidly and the rate of filtration slows down, presumably as the result of mutual interference of organisms. In any particular experiment at pressure x sufficient for the passage of the organism, the

concentration of *B. coli* in the filtrate gradually falls off as also does the rate of filtration, from a definite initial value, and this effect is attributed to the narrowing of the pores by adsorbed protein material. Hence another practical point observed in all experiments, except with 6·0% membranes when 2·5 c.c. samples were taken, has been to take always 5 c.c. filtrate samples, thereby eliminating any possibilities of variation due to inconsistent sampling.

B. prodigiosus.

This organism when grown on solid media is almost coccus-like in form—0·75 to 1·0μ in diameter—while its colonies on agar are red to reddish-purple in colour. These characteristics make it an extremely useful organism for control experiments and its filterability therefore is of considerable importance. For the purposes of the present study the organism was cultivated in agar slopes in the dark at room temperature. Some of a 48-hour culture taken on a platinum loop was dispersed in the saline/serum broth=10/1 medium as in previous experiments. The concentration of the organism was of the order 100 millions per c.c. The results are summarized in Table III and a graph showing the relative filterability of *B. prodigiosus* as compared with the other organisms studied is given in Fig. 6. The various filtrates were examined by making agar plate cultures and the concentration estimated from counts.

The results are quite analogous to those obtained with *B. coli*. *B. prodigiosus* filters somewhat more readily than *B. coli*, but the difference is not great.

Experiments were made with *B. prodigiosus* to determine the relationship between concentration of filtered organism in the initial suspension and that obtaining in the filtrate. The graph of results obtained with a 2·0% membrane at 40 cm. mercury pressure appears in Fig. 4. The rate of filtration falls off while the filtrate concentration increases, with greater concentration of the organism in the initial suspension. Under the conditions prevailing in the cited case the membrane effectively retains *B. prodigiosus* provided the concentration of the latter organism in the initial suspension does not exceed 10^3 per c.c.

B. bronchisepticus.

This micro-organism, which is one of the smallest of the identified bacteria, was discovered by Ferry (1915) during his researches upon canine distemper. He found that this organism would pass tested bacteriological filters and was led to include it among the filterable organisms. It may be cultivated readily in all ordinary media and hence it was considered that it would be very useful to compare its filterability through ultra-filter membranes with the behaviour of the other selected organisms. For the present experiments it was grown on agar at 37·5° C. and suspensions of a 48-hour culture were prepared in a manner exactly as in the case of *B. prodigiosus*, and the filtrates were examined by cultural test. The results are presented in Table IV.

This organism was found to filter more readily than *B. prodigiosus* which confirms the findings of Ferry, and furthermore is quite in keeping with microscopical evidence, for if the two organisms, *B. bronchisepticus* and

B. prodigiosus are simultaneously examined by the dark ground method, the former is seen to be definitely the smaller. Referring to Fig. 6 it will be seen that in a classification based upon filterability, *B. bronchisepticus* comes in the ordinary bacteria group with *B. coli* and *B. prodigiosus*, the filterable viruses as typified by bovine pleuro-pneumonia being much more readily filtered.

Bovine Pleuro-Pneumonia.

The bovine pleuro-pneumonia organism is generally recognized as a member of the filterable virus class and provides the only instance in which a virus has been successfully cultivated in artificial medium. It is readily grown in serum broth media, a vigorous culture exhibiting two distinct forms of the organism when examined microscopically by the aid of the best dark ground apparatus: (*a*) Spheres which come just within the present resolution limits (0·20–0·25μ, and (*b*) particles possessed of vigorous movement. Filtration experiments, therefore, are of utmost interest and significance, and this organism is of great value as a control in filtration experiments with filterable viruses, for which purpose it is used by the author. A detailed investigation of the morphology of bovine pleuro-pneumonia has been made by Smiles (1926). The growth of a serum broth culture at 37·5° C. is most vigorous at 3 days, at which age it contains spheres single or with one attached particle, and numerous free particles. In all experiments such a 3-day culture has been used, it being diluted with ten times its own volume of saline. The concentration of the organism was invariably of the order 500,000 per c.c. The filtrates were examined by plating out known volumes on serum agar in petri dishes and concentration estimated by counting colonies and comparing with dilutions of the original. The filtrates were also examined microscopically by the dark ground method in order to determine whether or not they contained the spherical form of the organism. The results obtained are summarized in Table V.

The particulate or granular form is clearly much more easily filtered than the spherical modification and can be separated from the latter by ultra-filtration in this way. On cultivation these particles give rise to spheres and the 3-day cultures exhibit the normal characteristics described above.

There is quite a considerable pressure period where the particulate form passes to the exclusion of the spherical and not until a definite pressure value obtains do the spheres appear in the filtrate. Comparing the pressure/permeability curve for the spheres (considered to be of diameter 0·2–0·25μ) with those for *B. coli*, *B. prodigiosus* and *B. bronchisepticus*, it appears to be misplaced when interpreting the curves in terms of size relationships of the organisms. This is without doubt attributable to the fact that whereas the pleuro-pneumonia spheres were estimated microscopically the presence of the other organisms was proved by the cultural test, which of course in the case of pleuro-pneumonia does not differentiate the particulate from the spherical form.

Here is a practical point of great importance in ultra-filtration work and one which has received all too little emphasis in the past, namely, the relative accuracies of methods used in filtrate examination and their bearing on the

interpretation of filtration results. One has only to consider for a moment a few of the outstanding methods in use to appreciate how easily results may be misconstrued, especially when the limiting permeability of the membrane is all important as is the case in all bacteriological experiments. The cultural test is an extremely sensitive one, even when allowance is made for the fact that the viability of the organism is not necessarily 100%. No ordinary analytical method can compare with it in delicacy. A point to be appreciated also is that results given by the cultural test will always be in terms of the maximum membrane permeability for the prevailing conditions.

As already stated the particulate form of bovine pleuro-pneumonia filters much more easily than the spherical modification and also than *B. bronchi-septicus* and *B. prodigiosus* (Fig. 6). The conclusion arrived at from the results of the present experiments in regard to the average size of the pleuro-pneumonia particle is that it is of the order 0·125–0·150μ, which is somewhat smaller than the value 0·20μ given by Barnard (1925), based on microscopical evidence.

Bacteriophage.

The phage used in these experiments was cultivated in broth cultures of *B. coli* at 37·5° C., the clear opalescent fluid remaining after the lysis of the bacteria being then diluted with 10 volumes of saline to which horse serum was added in order to make the medium comparable with that used in the other experiments.

The filtrates have been examined by inoculating broth cultures of *B. coli* and then plating out samples on agar after definite incubation periods. Wonderfully clear-cut results are obtained in this way, and by conducting control experiments with dilutions of the original a quantitative interpretation is possible by making counts of the sharply-defined lytic areas. The results recorded here are only qualitative or very roughly quantitative, the more detailed study of this interesting phenomenon being still in progress. The general filtration results are summarized in Table VI.

The phage has readily traversed all the membranes in the series. While the filtrates given by the more permeable membranes—below 3·0%—appeared not to differ appreciably in activity from the original unfiltered material, in the cases of membranes from the more concentrated collodions a gradual decrease in relative filtrate activity was manifest. The dotted line in Fig. 6 represents the approximate relative position of bacteriophage as compared with the filterabilities of the other organisms studied. Phage is clearly in order of size considerably smaller than bovine pleuro-pneumonia. Several investigators have assigned values for the size of bacteriophage. Prausnitz (1922), using de Haen membrane filters, compared its filterability with that of colloidal silver and concluded its size to be of the order 20$\mu\mu$; von Angerer (1924), using methods of refraction, estimated its size as 30$\mu\mu$; while Eliava and Suarez (1927) experimenting with Bechhold membranes gave the value 5$\mu\mu$. Again, Levaditi and Nicolau (1923) as the result of filtration through collodion sacs came to the conclusion that phage was of the same order of size as the viruses of rabies, vaccinia, and encephalitis lethargica. There is no doubt that its size is well within the truly colloidal zone, and as emphasized

by Alexander (1927) any assigned size value deduced from filtration experiments must of necessity be very approximate in view of the numerous factors —surface phenomena—coming into filtration processes with systems of this fine order of dispersion.

All the membranes described and used in these experiments were readily permeable to oxyhæmoglobin, albumoses and peptones, while serum albumin and globulin as indicated by turbidity tests on boiling filtrates adjusted to pH 4·6–5·0, passed all membranes up to and including 4·0% with but little alteration in concentration, but in the case of 5·0% and 6·0% membranes the filtrate concentration was considerably less than the original.

DISCUSSION OF RESULTS.

Surveying the results obtained their implications are fairly well defined. Ultra-filter membranes can be prepared from glacial acetic acid collodion to be of distinct service in bacteriological studies, but the precise filtration processes are governed to a large extent by the conditions of experiment. That pore size is the chief factor is clearly shown in Fig. 6, representing the relative filterabilities of the various organisms filtered. The positions of the curves are in the generally recognized order of size of these organisms, although not as markedly separate as one might wish. However, while not permitting the certain separation of members of the ordinary bacteria group, *e. g.*, *B. prodigiosus* from *B. coli*, the following can definitely be accomplished : (*a*) Separation of red corpuscles from bacteria; (*b*) separation of bovine pleuro-pneumonia from bacteria; (*c*) separation of bacteriophage from bovine pleuro-pneumonia and therefore from bacteria.

It may reasonably be hoped, therefore, that the use of ultra-filtration methods will be of positive value in investigations appertaining to filterable viruses. A dogmatic statement upon this point is not warranted in view of the evidence already outlined. Thus, in each particular instance, the medium constituents have to be gauged in terms of their filterability, and furthermore it will have been noticed that filtration is accompanied by a considerable dilution of the organism. The exact value of this dilution effect is dependent upon filtration conditions. This is a point of fundamental significance when translated to experiments with viruses. The presence of the viruses in filtrates can only be detected by animal experiments, all attempts to cultivate the viruses in artificial media having so far failed. Hence the unit is a relatively coarse one—the minimum infective dose—and unless the initial material has a high infectivity value there is a distinct possibility that the virus, although present in the filtrate, is in amount insufficient to produce the disease. Such points as these have to be carefully considered in ultra-filtration with filterable virus and can only be effectively countered by systematic control experiments.

It is important to realize that the data furnished by the present experiments apply strictly only for the particular membranes used and the experimental conditions prevailing. Employing a different nitro-cotton, the membrane characteristics will certainly vary from those obtaining here. By standardizing the membranes in the manner outlined, one is able to gauge their filtration capabilities only for particular experimental conditions. Departure from these

specified conditions of standardization as must inevitably occur in applying ultra-filtration methods to concrete problems, makes control experiments essential. One of the organisms used in the standardizing experiments must be suspended in the new medium—may be tissue extract as frequently employed in virus experiments—and parallel filtration conducted. It will invariably be found that considerably higher pressures are necessary to effect the passage of the control organism than was experienced under the standardizing conditions which of course are exceptionally favourable ones. The presence of mucin and other tissue proteins diminishes the filterability of a suspended organism. For example, in filtering saline extracts of Rous sarcoma tissue a 1·5 % membrane has been found impermeable to *B. prodigiosus* throughout its pressure range.

A further question arising concerns the mechanism whereby bacteria traverse the membrane. It is most certain that there are pores of size appreciably larger than the average value calculated from the rate of flow of water measurements. It may reasonably be concluded, therefore, that since several of the organisms filtered are known to be larger than the average pore size of membranes permeable to them, that here is in reality an indication of the maximum pore size. This is probably the correct interpretation of the results. However, the fact must not be overlooked that bacteria are not of non-deformable structure, and hence the possibility exists that they may be able to accommodate themselves by slight deformation to traverse channels of somewhat lesser diameter than what is normally their own. The certain parallelism to be observed between the filtration curves (Fig. 6) for bacteria like *B. coli* and *B. prodigiosus* (when passing in appreciable concentration, or, in other words, when the membrane is generally permeable) and that for red corpuscles, which latter are unquestionably deformed in traversing the membrane, might well be regarded as evidence in favour of analogous processes.

A complete answer to this permeability question involves the nature of the structure of these membranes. Here two possibilities occur:

(1) The gel may simply coat each individual cellulose fibre with a film which varies in thickness with the concentration of the collodion being a function of the viscosity.
(2) The cellulose fibres play their part as primarily intended, namely, as a reinforcement to the otherwise continuous gel film.

The former cannot be upheld since microscopical evidence shows the gel filling the interstices of the paper. The ruling membrane structure, therefore, is essentially that of the gelatinous film itself subjected to modification due to distortions resulting from the presence of the imbedded mat of interlacing cellulose fibres. The fact that these membranes are less regular and uniform than pure gel membranes is to be attributed to two factors: (*a*) The influence of the fibres of the filter-paper which present no geometrical uniformity of arrangement themselves, and (*b*) to the inherent differences between the films as used here and true gel films as has already been indicated in an earlier paragraph. The more fundamental point at issue—the question of gel structure—will be considered in a later paper.

SUMMARY.

1. A simple and convenient method for preparing impregnated acetic acid collodion membranes with uniformity of thickness throughout the complete concentration range is described. Also a suitable technique for preparing such membranes in sterile condition by use of an ultra-violet light chamber is outlined.

2. The applicability of these membranes in bacteriological studies has been investigated, studies having been made of the filterability of red blood-corpuscles, *B. coli*, *B. prodigiosus*, *B. bronchisepticus*, bovine pleuro-pneumonia and bacteriophage. The numerous determining factors and essential practical points to be observed in connection with filtration of such systems are emphasized.

3. The size of the particulate phase of the bovine pleuro-pneumonia organism has been estimated as 0·125–0·150 μ while that of the bacteriophage is very much smaller.

4. Evidence that ultra-filtration methods should prove of positive value in filterable virus studies is definitely established. However, intelligent cognizance of determining factors in surface phenomena is imperative since the conception of filtration as consisting simply of a sifting process with relative pore size the sole controlling factor is wholly inadequate when dealing with truly colloidal and sub-colloidal systems.

In conclusion, the author expresses thanks to Mr. J. E. Barnard, F.R.S., for his kindly interest in the work and his valued advice in the microscopical studies; and also to Mr. F. V. Welch for his assistance in the bacteriological cultural tests.

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FILTER AND CENTRIFUGE EXPERIMENTS WITH NEUROVACCINE.

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Received for publication February 6th, 1929.

In a previous paper (Bland, 1928) the author demonstrated his inability to filter vaccinia virus in the form of suspensions of guinea-pig vesicle material through membranes of collodion which would permit the passage of the least dispersed of the serum proteins. This observation suggested that the particles of the virus might be larger than is sometimes supposed, and it was supported by the results of centrifugalization experiments, in which it was found possible to concentrate the virus, in the form of diffusates from guinea-pig pads, by spinning at about 6000 r.p.m. for 2 hours.

These filtration experiments conflicted with the findings of Levaditi and Nicolau (1923), who had succeeded in filtering neuro-vaccine through their collodion membranes with little difficulty. The author suggested that this conflict of evidence might be explained in two ways: either the particles with which the virus is associated are of different sizes in skin and brain material, or the membranes of Levaditi and Nicolau let through the virus because they leaked—a possibility which those authors, apparently, did not investigate.

The present paper deals with an attempt to clear up this question by conducting a series of experiments with neuro-vaccine similar to those reported in the previous paper. It will be seen that the results favour the second of the two explanations.

MATERIALS AND TECHNIQUE.

These were essentially similar to those used in the previous paper and differences only will be recorded here.

Virus.—The strain was a neurovaccine kindly provided by Prof. McIntosh, and is propagated in rabbits by intra-cerebral passage. The animals develop an encephalitis, usually within 48 hours of inoculation, which is manifested by weakness, paresis, tremor, head-nodding and general convulsions; to this they succumb in 72–96 hours.

As soon as is practicable after death the brain is removed with sterile precautions and is stored whole, in the ice-chest, under paraffin without glycerol or any other fluid, as is described by Perdrau (1927) for the preservation of herpes virus. From this stock pieces are dug out with a sterilized spud as occasion requires.

The virus was used for filtration and centrifugalization in four forms:

(1) Emulsions in M/50 phosphate buffer, pH = 7·6.
   (*a*) Whole emulsion; (*b*) supernatant after light centrifugalization.
(2) Diffusates into—
   (*a*) Broth; (*b*) M/50 phosphate buffer, pH = 7·6.

These types of virus will be referred to in the tables which follow as Ea, Eb, Da and Db.

The virus is titrated by the intradermal injection of 0·1 c.c. of falling dilutions in the shaven skin of the back of rabbits. The usual type of reaction is observed after such an injection, except that with the stronger concentrations of this virus there occurs a good deal of necrosis of the central area of the take at about the fourth or fifth day which proceeds to the formation of a tough, black, horny scab that is very adherent and may persist for many weeks.

A difficulty was experienced early in the work in the appearance, on the fifth or sixth day, of a generalized eruption of small shotty papules which sometimes showed a hæmorrhagic areola and necrosis of the central area, and was often accompanied by snuffles and conjunctivitis. This eruption rendered it impossible to take readings of the titrations after the fourth or fifth day.

Membranes were made of 3% collodion, and were used both plain and treated for 24 hours with 1/10 normal rabbit serum.

Filtration.—The Seitz apparatus was used with negative pressures from 100 to 150 mm. Hg. in different experiments.

FILTRATION EXPERIMENTS.

A series of 7 experiments was done, following a plan almost identical with that described in the previous paper. The filtration of virus was again compared with that of the protein in the same emulsion. The titre of the virus and filtrates was ascertained by intradermal titration, but for the estimation of protein the precipitin reaction was not used. Instead a series of decimal dilutions of the unfiltered material was made and to each was added an equal quantity of 20% salicyl-sulphonic acid; the filtrate was then treated in the same way, and the degree of flocculation or opacity matched with one, or between two, of the dilutions of unfiltered virus suspension.

Each membrane was tested immediately after use by filtering through it a suspension of *B. paratyphosus* B. Membranes which allowed the passage of the bacillus were rejected.

A study of Table I in which these experiments are summarized, reveals the fact that while all the membranes let through some protein (in the case of treated membranes a considerable amount), virus was detected in the filtrate from only one. This doubtful trace take, which consisted of two small nodules widely separated instead of the usual single nodule, did not make its appearance until a day after the highest dilution of the virus titration had shown a positive reaction. Two days after its appearance, the sixth after inoculation, generalization took place, but was not very marked, and consisted of only a few small nodules similar to that composing the doubtful take. It is possible, indeed probable in view of the consistently negative results of the other experiments, that this take was an early manifestation of generalization, the other early stages of which passed unobserved.

This series of experiments appears to show clearly that neurovaccine virus cannot pass collodion membranes. It is possible to object, however, that in its passage through such a membrane the whole of it may be adsorbed even to the extent of 15,000,000 M.V.D., and that none may be left to reach the filtrate. Further, it may be said that although by treating a membrane with

TABLE I.—*Filtration Experiments.*

Experiment.	Type and strength of virus emulsion.	Titre of virus.	Degree of dilution allowed for in test.	Filtrates from treated membranes.		Filtrates from plain membranes.		Number of membranes in each test.
				Amount of protein.	Presence of virus.	Amount of protein.	Presence of virus.	
1	E b. 10%	1/100,000	1/10,000	1/1–1/10	0	1/100	0	2
2	E a. 1%	1/100,000	1/1000	—	—	1/10,000	0	1
3	E b. 10%	1/100,000	1/10,000	1/1–1/10	0	—	—	1
4 1	E b. 5%	1/1,000,000	1/50,000	1/5	0	1/500	0	4
4 2	„ 1%		1/10,000	1/1–1/10	? trace	1/1000	0	
5 1	E a. 2%	1/1,000,000	1/20,000	—	—	1/2000	0	3
5 2						„	0	
5 3						„	0	
6 1	E b. 2%	1/1,000,000	1/20,000	1/2–1/20	0	—	—	2
6 2				„	0			
7 1	E a. 1%	1/1,000,000	1/10,000	1/100	0	—	—	3
7 2				„	0			
7 3				„	0			
8 1	D a. 1%	1/1,000,000	1/10,000	—	—	1/2	0	1
8 2						more than 1/2	0	
8 3						„ „ „	0	
9 1	D b. 10%	1/10,000	1/1000	—	—	1/10–1/100	0	1
9 2						1/1–1/10	0	
9 3						more than 1/1	0	
9 4						„ „ „	0	
9 5						„ „ „	0	

In columns 5 and 7 the protein content has been expressed as the dilution of the virus emulsion with which the filtrate matched; where two fractions occur, *e.g.* 1/1–1/10, this means that the filtrate fell between these tubes.

serum we can satisfy its affinity for serum proteins, its affinity for virus may remain unchanged. In order to answer these objections two experiments were made in which successive quantities of virus were filtered through the same membrane. If it be granted that such a procedure would satisfy the adsorbing power of the membrane for virus, these experiments show that it is not adsorption which prevents the virus appearing in the filtrates. These experiments are Nos. 8 and 9 in Table I, and the full protocol of No. 9 is given below.

EXPERIMENT 9.—*Saturation of Membrane with Virus.*

Virus.—A 10% phosphate diffusate of brain. Membrane 3% collodion, washed 4 days.

Filtrations.—3 c.c. virus each time at 145–150 mm. Hg. pressure.

(1) Took 10 mins. Filtrate almost colourless.
(2) „ 23 „ Colour about half that of virus.
(3) „ 32 „ „ almost equals that of virus.
(4) „ 39 „ „ „ „ „
(5) „ 44 „ „ „ „ „

Protein titration: 18 drops of each test + 9 drops salicyl-sulphonic acid. Degrees of flocculation or opacity by + signs; no opacity, −.

Dilutions of virus.					Filtrates.				
1/10	1/100	1/1000	1/10,000	1/100,000	1	2	3	4	5
++++	++	±	∓	−	+	+++	+++++	+++++	+++++

Virus titration: 0·1 c.c. of each dilution intradermally. Seventh day readings. No generalization. Degrees of take by + signs; no take, −.

Dilutions of virus.				Filtrates.				
1/100	1/1000	1/10,000	1/100,000	1	2	3	4	5
++	+	±	−	−	−	−	−	−

Leakage test.—The filtrates did not contain *B. paratyphosus* B.

N.B.—In this experiment the dilutions in the tables are dilutions of *original brain.*

CENTRIFUGALIZATION EXPERIMENTS.

A series of six experiments was done in which the virus was spun at about 6000 r.p.m. for 2–2½ hours. At the end of this period a sample (0·5 c.c. or less) was withdrawn from the upper layers of the supernatant fluid. The middle layers were then carefully pipetted off and discarded and the bottom 0·5 c.c. or so were washed up and down on the deposit. These two samples, supernatant and deposit, were then titrated in the usual way along with a sample of the original virus suspension.

The results of these experiments showed that in every case centrifugalization caused a tenfold diminution of the virus content of the supernatant as compared with the original virus. On the other hand, this diminution was not counter-balanced by a constant tenfold increase in the deposit. Indeed this occurred in only two experiments. In the rest the titre of the deposit was identical with that of the original virus, but the size of the takes obtained

with the deposit was greater than those of the virus titration, and this is an indication that some concentration had in fact occurred. Further, brain emulsions or diffusates contain varying quantities of lipoids which rise to the surface during centrifugalization. This centripetal movement of lipoid particles probably interferes with the centrifugal movement of virus particles, and it is possible that had a sample been taken from some part of the middle layers a greater concentration might have been found than was present in the deposit.

This series of simple centrifugalizations suggested that the virus was large enough to be spun down by centrifugal force, but it was thought that the demonstration was not conclusive; it might be objected that the small concentration obtained was due to the adsorption of the virus on to the larger particles of tissue which might still have been present even in the diffusates and lightly spun emulsions which were used for this investigation. Such a criticism could be met were it possible to show that the virus remaining in the upper layers of a centrifugalized sample could be further concentrated by recentrifugalization. Four experiments were actually done on such a plan.

Ten per cent. broth and phosphate diffusates of brain were centrifuged from 1–2½ hours at 6000 r.p.m. At the end of this time 0·5 c.c. of the upper layers was removed, the whole of the middle portion, some 5 c.c., was carefully pipetted off into another centrifuge tube and the deposit was treated as before. The middle layers were now thoroughly mixed, and after a small sample had been withdrawn for titration they were again spun for 2–2½ hours at 6000 r.p.m. When this second spinning was completed samples of supernatant and deposit were taken as before. All the five samples, supernatants 1 and 2, deposits 1 and 2, and middle layers, were then titrated along with the original diffusate.

The concentrations achieved by these experiments are summarized in Table II. They leave no doubt that the recentrifugalization of the supernatant from a centrifugalized diffusate achieves a further concentration of the virus. It is scarcely credible that this result can be due to the deposition of fragments

TABLE II.—*Concentrations of Virus Obtained by Double Centrifugalization.*

	Concentration of virus by first centrifugalization.			Concentration of middle layers by second centrifugalization.			
Number of experiment.	Number of times deposit 1 exceeds virus.	Number of times deposit 1 exceeds supernatant 1.	Number of times virus exceeds supernatant 1.	Number of times deposit 2 exceeds virus.	Number of times deposit 2 exceeds middle layers.	Number of times deposit 2 exceeds supernatant 2.	Number of times middle layers exceed supernatant 2.
1	1000	1000	>10	100	100	100	>10
2	10	100	10	10	>10	10	10
3*	10	10	?	10	10	10	?
4	>10	10	10	>10	100	1000	10

* In this experiment the uncentrifugalized virus, middle layers, supernatants 1 and 2 gave no take at 1/10, the lowest dilution employed. Deposits 1 and 2 gave takes at 1/10, but not at 1/100 Experiments 1, 2 and 3 were done with broth diffusates 10%; Experiment 4 with a phosphate diffusate 10%.

of tissue or cells in which the virus is entangled or adsorbed. Such fragments would be thrown down by the first period of spinning, and this second concentration must be caused by the action of centrifugal force on the virus itself, or at least on the particles with which it is associated.

SUMMARY

Neurovaccine, then, is a variety of vaccinia virus which responds to filtration and centrifugalization exactly as does the guinea-pig skin virus which was used for the previous series of experiments, and there is no reason to suppose that there is any great difference of size between the particles which form these two strains. Neurovaccine will not pass membranes of 3% collodion whether these are used plain, treated with serum or saturated with virus, membranes some of which let through a large proportion of protein. Further, centrifugalization at the comparatively low speed of 6000 r.p.m. for 2 hours is capable of throwing down the virus in part, nor is there any indication that it is not the virus itself which is acted upon. Such a virus, or the particles with which it is associated, must be of greater size than is sometimes supposed, and may be within the limit of microscopic visibility.

These results do not support the findings of Levaditi and Nicolau. It must be borne in mind, however, that it is difficult to make collodion membranes of a constant degree of permeability, and since these workers did not use the same membrane for the virus filtration and the filtration of the test substance and neglected to test their membranes for microscopic leaks, their experiments lack the controls necessary for their evaluation.

The author's thanks are due to Prof. McIntosh for kindly providing him with the strain of neurovaccine which was used in this work.

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TETANUS.—VIII. THE POSITIVE LIMIT OF OXIDATION-REDUCTION POTENTIAL REQUIRED FOR THE GERMINATION OF SPORES OF *B. TETANI IN VITRO.*

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Received for publication January 10th, 1929.

In a previous paper (Fildes, 1927) it was contended that the failure of tetanus spores to germinate when inoculated alone into guinea-pigs was due to the tension of oxygen in the subcutaneous tissues being too high to permit germination.

In order to support this concept it is necessary to show first what degree of anaerobiosis is necessary before tetanus spores can germinate, and secondly how this state compares with that in the normal tissues. In the present paper the former subject is dealt with.

INDICATORS OF OXIDATION-REDUCTION POTENTIAL.

According to the view of Mansfield Clark (1924) and to obvious extensions of it the term "anaerobe" as applied to a micro-organism implies that it requires for growth a certain reducing intensity in its surroundings. The absence of molecular oxygen is not the essential factor except in so far as oxygen under ordinary circumstances of growth tends to prevent the necessary degree of reducing intensity from developing. Should, however, the reducing intensity of the surroundings be great, the desired conditions may be attained even in the presence of air, and growth of an anaerobe may then take place.

Mansfield Clark and his collaborators have up to now confined themselves almost entirely to the study of the chemical aspects of oxidation-reduction and have allowed themselves few excursions into biology. Nevertheless, these excursions in so far as they relate to anaerobiosis seem to be of outstanding interest, and to offer a most satisfactory explanation of the phenomena observed by the bacteriologist. It therefore seems a little curious that hitherto the work of Mansfield Clark and his collaborators has not attracted the attention one would have expected. In a recent work (Jordan and Falk, 1928), although a short chapter by Clark is included, it is followed by another chapter on anaerobiosis, in which there is no mention of his views.

The details of the work of Clark and his collaborators, Cohen, Sullivan, Gibbs and Cannan, were published in the Public Health Reports of the United States Public Health Service from 1923 onwards. The first ten papers (1923 to 1926) were reprinted recently as the 'Hygienic Laboratory Bulletin No. 151.'

* Working under grants from the Medical Research Council.

In these papers Clark and his collaborators discuss, *inter alia*, the theoretical considerations underlying the method of estimating reducing intensities by means of indicator dyes. It is true that these dyes have been used for some years by bacteriologists to indicate degrees of reducing intensity, but hitherto there has been no clear understanding of their possibilities or limitations. It has of course been known that "good anaerobic conditions" are those in which methylene blue will remain reduced; that thionin, methylene blue or safranin are progressively more difficult to reduce, *i. e.*, they require to be subjected to progressively more active reducing conditions; that having been reduced they become oxidized again in the reverse order. Thus these three dyes have indicated increasingly pronounced degrees of reducing intensity.

Observations of this sort are now put upon a more satisfactory basis.

In the case of soluble systems which can undergo reversible oxidation and reduction under different external conditions (such as certain dye stuffs in reversible equilibrium with their leuco compounds), an electrode of a noble metal immersed in a solution containing a mixture of the reduced and oxidized form at a given pH will show a definite electrical potential difference, varying in a regular way with the proportion of reduced to oxidized form. By combining this half cell with a second half cell of known E.M.F., the total potential difference may be measured, and the difference of potential between the oxidation-reduction system and the electrode immersed in it may be calculated. As his second half-cell Mansfield Clark selects the "normal hydrogen electrode" for convenience in theoretical treatment (although it is not directly used in the practical measurement), and gives it, arbitrarily, a zero potential. He is now able to express in volts the oxidation-reduction potential ("Eh") of any given system containing a definite ratio of reductant to oxidant.

The chemical significance of Eh is that it is a direct measure of the free energy of hydrogenation of the oxidant of the system involved. That is to say Eh may be taken as a measure of the "reduction intensity" of the system compared with that of an atmosphere of hydrogen (in the presence of a catalyst). Moreover since oxidation and reduction are complementary terms for a single chemical process, Eh becomes a measure of the oxidation intensity of the system in the sense that a system of high reduction intensity is one of low oxidation intensity and *vice versâ*.

Eh is defined (at constant pH) by the equation—

$$\mathrm{Eh} = \mathrm{E'o} - 0{\cdot}062 \log \frac{a}{100 - a} \text{ (at } 37^\circ \text{ C.)}.$$

where E'o is a constant characteristic of the particular system and $a = \%$ reduction. Once E'o has been determined it is possible to calculate Eh for any desired value of a, the correction to be applied being the same for all oxidation-reduction systems studied. The value of this lies in the fact that Clark and his colleagues have determined the E'o of a number of organic systems suitable as colorimetric indicators. The oxidants of these systems are coloured and their reductants are colourless. Thus by the addition of one of these dyes to any unknown system and the determination of the percentage of colour remaining, the value of a and hence Eh may be arrived at.

In Table I are given the values of a series of Clark's indicators when 98% reduced, *i. e.*, when almost colourless and in Table II the corrections to be applied to these to correspond to other degrees of reduction.

TABLE I.

Name.	Abbreviation.	Eh.
Phenol indo 2, 6 dibromophenol	Indophenol 2	+ 0·166
1-naphthol 2-sulphonate indo 2, 6 dibromophenol	Indophenol 1	+ 0·067
Thionin	...	+ 0·012
Methylene blue	M.B.	— 0·040
Indigo tetrasulphonate	Indigo 4	— 0·097
Indigo trisulphonate	Indigo 3	— 0·132
Indigo disulphonate	Indigo 2	— 0·176
(Hydrogen electrode		— 0·420)

TABLE II.—*Factors to be Added (Algebraic) to Figures for 98% Reduction to Correct for other Degrees of Reduction.*

99% = — 0·009	60% = + 0·046
95% = + 0·013	50% = + 0·051
90% = + 0·022	25% = + 0·065
80% = + 0·033	1% = + 0·111
70% = + 0·04	

As already stated, the value of Eh depends upon pH, those given in Table I being for pH 7·0. The figures for other pH values are shown in Fig. 1. It will be gathered from this figure that reduction of methylene blue takes place at a more negative Eh in an alkaline medium than in an acid. Thus at pH 8·0 the reduction of methylene blue will indicate an Eh negative to that indicated by reduction at pH 7·0. Again, if indophenol 2 is added to a fluid of a certain potential, *i. e.* Eh + 0·2, both at pH 6 and pH 8, it will be colourless in the former case and blue in the latter.

It should be pointed out that the *plus* or *minus* signs attached to Eh have no significance in relation to oxidation or reduction. Eh 0 is merely the value assigned to the hydrogen electrode at pH 0. At pH 7·0 this value has fallen to — 0·420 (Fig. 1), and the values of several dyes have fallen below zero and so carry the negative sign, although they are all positive to the electrode.

GENERAL PROCEDURE.

The present paper gives an account of an attempt to utilize Clark's indicators for the purpose of measuring the reducing intensity required to be present in the surroundings of a tetanus spore before it can germinate.

As Clark has repeatedly pointed out, the chemical basis upon which the use of the indicators depends is in need of further investigation, and it is not to be supposed that results obtained with the dyes will have the precision of

the electrode measurement. Nevertheless it seems clear that, with proper precautions, broad indications may be obtained which are sufficient for the

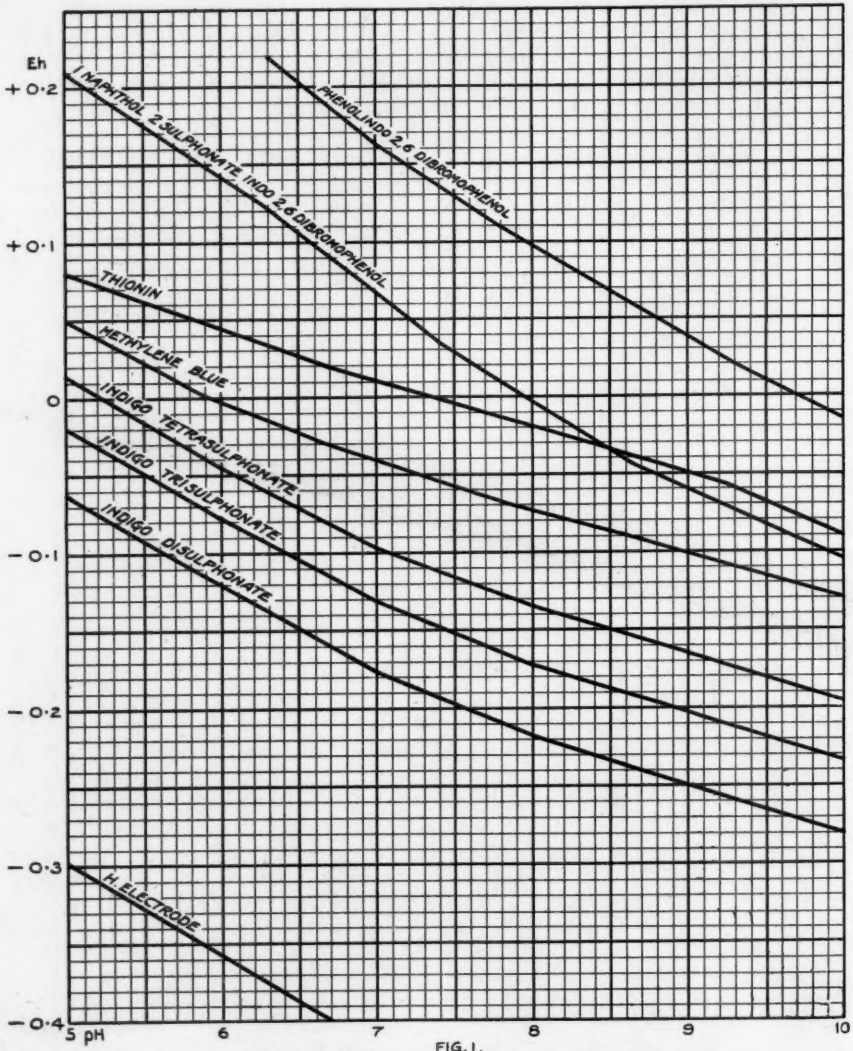


FIG. 1.
Eh INDICATED BY 98% REDUCTION OF DYES AT DIFFERENT pH.

purpose now in view. It will be noticed that this purpose is to study the state of the surroundings of a spore *before* it germinates, and not the possibly more

highly complicated condition induced by this germination. Previous work in bacteriology has been confined to the latter object.

The general procedure adopted at first was to add the indicators to a culture medium, inoculate it with spores, and place the mixture at 38° C. with exclusion of air. Under these conditions certain dyes would become reduced and thus indicate that the medium had acquired a negative potential of a certain degree. At this moment the culture was opened and examined for germination. Experiment 1 shows an example of this procedure.

Experiment 1.—Meat infusion broth containing 1% glucose (= glucose broth); pH 7·6*; 10 c.c. in test-tubes 6 × $\frac{5}{8}$ inch; 30 tubes placed in boiling water for 10 minutes and then into cold running water for 2 minutes; arranged in 6 groups of 5; to one group add no dye, to another add thionin (Appendix I, p. 169), to another M.B., and so on (indigo 4, 3 and 2); to all, except one tube of each dye, add 1 drop of the spore suspension in use (Appendix II, p. 170), and run in $\frac{1}{2}$ inch vaseline at about 140° C., which has been heated to 180° C. Stand all tubes in cold water until the vaseline is set; agitate the tubes in hot water until they are at about 38° C. and invert them to mix; incubate at 38° C. Observe the time.

Complete reduction of thionin both in the inoculated and uninoculated series took place in $\frac{3}{4}$ hour (Appendix III, p. 170); of M.B. in $1\frac{3}{4}$ hour; of indigo 4 in 2 hours. One tube without dye and with the various dyes opened in 4 hours: no germination in any (Appendix IV, p. 170). Again in 5 hours: indigo 3 not yet reduced; no germination. Again in 6 hours: germination now present in all tubes; indigo 3 nearly reduced; indigo 2 slightly reduced (Appendix V, p. 171).

On reference to Fig. 1 these states of reduction of indigo 3 and 2 indicate at pH 7·6 an Eh of about −0·14.

From such an experiment, however, it is clearly not legitimate to conclude that this Eh −0·14 is a necessary factor in the germination of the spores, unless the lag of germination depends upon Eh alone. But this obviously is not the case. There are, of course, other factors in lag, nutritional, pH and so forth, and these factors may prolong the germination time so much that a medium in which the spores are suspended drifts to a more negative Eh than is necessary for germination before the spores have had time to germinate.

Thus Experiment 1 indicates that Eh −0·14 (pH 7·6) is adequate for germination, but a more positive might be equally adequate.

In experiments of this sort, therefore, it is easy to demonstrate that a certain reducing intensity is effective, but to prove that it is essential entails proving a negative—namely that the spores will not germinate under any conditions at a more positive reducing intensity.

The only available method of demonstration in such a case is to show that growth did not take place on the positive side of a certain dye, although every effort was made to induce it. These efforts consisted in holding the Eh of the medium for a long time in various zones on the reducing scale by different methods—in other words, in prolonging the time of reduction so much that the spore must have had excessive time to overcome any objection it might

* Throughout this work pH has been estimated by the method of Gillespie (Laidlaw *et al.*, 1927).

have to germination (lag) other than that due to inadequacy of the Eh When germination does not take place under these conditions the evidence is strongly in favour of the conclusion that the reducing intensity is not negative enough.

PRELIMINARY EXPERIMENTS.

Since the problem depends upon the relation between the time taken by a medium to attain a certain reducing intensity and the time of germination (lag), it is necessary to know the measure of the reducing intensity of various media, and also the lag under different conditions. With this information before us it will be possible to select a medium in which the longest time will be required to achieve a high reducing intensity, and in which the "lag" of *B. tetani* will be short. In this medium optimum conditions will be present to induce *B. tetani* to germinate at the most positive Eh possible.

Reducing Intensity of Media.

The course of development of a reducing intensity in a medium was studied as follows:

Experiment 2.—Applicable technique as in Experiment 1.

A series of 7 tubes of medium taken, and placed in water at 100° C. for 10 minutes and cold water for 2 minutes. Each then tinted to a minimum degree with one of the indicator dyes. The series then displayed in front of a white card in the anaerobic jar and the contents mixed by shaking after establishment of anaerobiosis. Note the time and place in the incubator. Five different series for each medium were thus put up, starting at a different time, and the time of reduction of the dye in each tube noted. The curves in Fig. 2 are to some extent composite, since they are based upon times obtained from the five series considered as one, it being impossible or inconvenient to be present to make observations continuously. It is not claimed that they are accurate to a high degree. Variations are of course observed with different batches of media, and even with the same batch used at different times and subjected to slightly different conditions. In 14 different experiments, however, the same general course was noted in the 3 media illustrated.

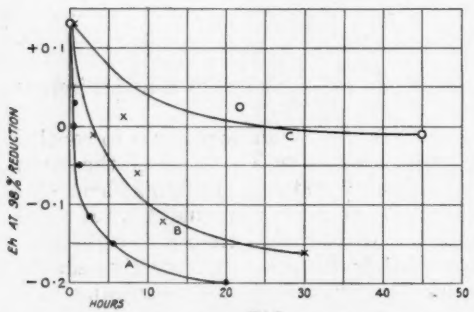


FIG. 2.
EFFECT OF CONSTITUTION OF MEDIUM ON RATE OF DRIFT OF Eh.
A. = GLUCOSE BROTH pH 7·5 INCUBATED AT 38° C. = • •
B. = ORDINARY BROTH pH 7·7 " " 40° C. = × ×
C. = PEPTONE WATER pH 7·6 " " 39° C. = O O

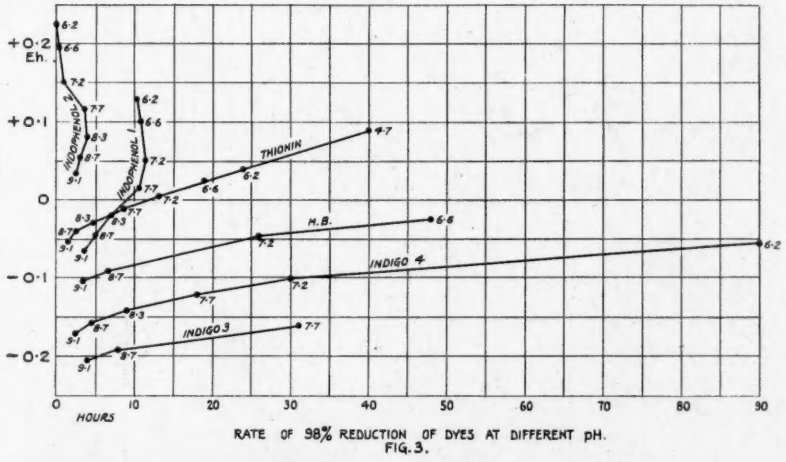
The initial Eh in each case is determined from the fact that indophenol 2 became reduced immediately after mixing. It will, however, be understood

that the real Eh may have been more positive than indicated. In each case also the curve has been drawn through the zone governed by indophenol 1 and M.B. before reduction of the dyes. As noted in Appendix V (p. 171), each of these dyes delays the drift of a medium in which they are incorporated. Further, the presence of the other dyes accelerates this drift, and therefore the curves cannot be taken as showing the exact course of drift of a medium alone without incorporated dye. Nevertheless it may be assumed that the 3 media illustrated would behave approximately as shown if their course of drift were measured electrically without added dye.

The curves show that glucose broth attains a marked negative Eh with great rapidity and then the drift becomes slower. Peptone water also drifts more rapidly at the beginning of incubation than at the end, but it never attains an Eh as low as glucose broth. Ordinary broth assumes an intermediate position. Other intermediate positions will be taken by mixtures of different proportions of ordinary broth and peptone water.

The Effect of pH on Drift.

pH has a marked effect upon the rapidity with which media attain a negative Eh. This is shown in the case of ordinary broth in Experiment 3.



RATE OF 98% REDUCTION OF DYES AT DIFFERENT pH.
FIG. 3.

Experiment 3.—Applicable technique as before.

7 tubes of broth, boiled and cooled; pH (at room temperature) adjusted by adding drops of N/5 acid and alkali as previously determined and then a minimal quantity of one of the seven dyes added to each; anaerobe jar, etc. The time of reduction of the dye at each pH noted and plotted against Eh (Fig. 3). As before, the curves for thionin, M.B. and the indigos are compounded from five different series.

If, now, lines are drawn through similar pH points, curves as shown in Fig. 4 are obtained. Several explanations are, however, required.

The start of each curve is drawn, probably erroneously, from the same point in all cases. This point, the initial Eh of the medium, is determined by the fact that indophenol 2 reduces immediately at pH 6·2 when the tubes are mixed, while the other tubes containing the dyes are coloured, *i. e.* the Eh of these tubes is above the line of indophenol 2 in Fig. 1.

The pH points on the lines of indophenol 2, indophenol 1 and M.B. are neglected. These tend as noted before to be too much to the right owing to delay in the drift with these dyes. Where observations have not been available to direct the curves, these have been carried through the presumed point. The figure is therefore schematic. The actual course of the curve as indicated by observations is shown dotted for pH 7·7.

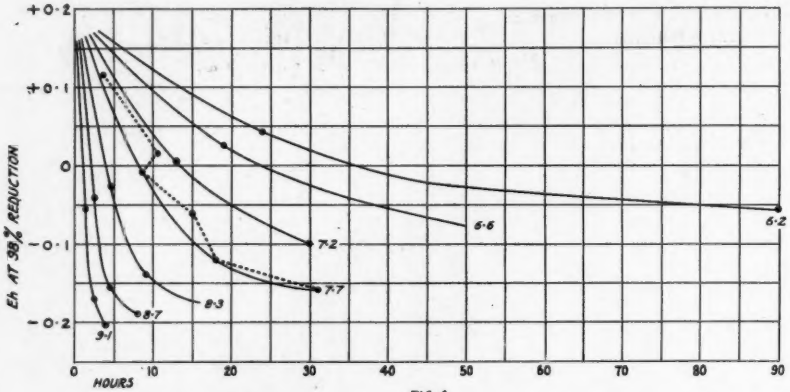


FIG. 4.
EFFECT OF pH ON THE RATE OF DRIFT OF A MEDIUM.

This figure shows that the rate of drift is increased by increase of pH and the ultimate Eh attained is more negative. The same effect is noted in the case of glucose broth and peptone water.

If, now, it is desired to select a medium which will maintain itself as long as possible at a positive Eh, it is clearly necessary to avoid a highly reducing medium such as glucose broth at a high pH, and to favour a medium such as broth or peptone water at a low pH.

The Effect of Reducing Intensity of the Medium on the Observed Eh of Germination.

The effect of two media of this sort upon the observed Eh for germination is as would be anticipated, namely in peptone water at pH 7·4 germination takes place at a much more positive Eh than in glucose broth at pH 7·8 or in ordinary broth at pH 7·6.

Experiment 4.—Technique as before, particularly as in Experiment 2, but each jar contained 2 series of tubes, one incubated with spores and the other without. In such a case the reduction of the dye in each pair of tubes takes place approximately at the same time (*cf.* Appendix VI, p. 171), until at a certain

point a wide divergence is noted. This divergence is due to germination of the spores (as may be confirmed if the jar is opened, when divergence is noted), and thus the approximate Eh of germination may be deduced (Fig. 5).

The Lag of B. tetani.

It having been determined that the requisite conditions of the medium are slight reducing intensity and low pH, it is now necessary to consider how these and other factors affect the second requisite, *i.e.* optimum conditions apart from Eh for early germination, so that this may take place at a point as positive as possible before the medium has drifted to a more negative Eh.

The lag of an anaerobic organism in culture must clearly depend upon several factors, among these being—

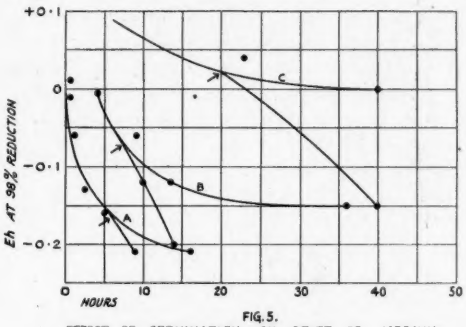


FIG. 5.
EFFECT OF GERMINATION ON DRIFT OF MEDIUM.
A = GLUCOSE BROTH pH 7·8
B = ORDINARY BROTH pH 7·6
C = PEPTONE WATER pH 7·4
SMALL DIFFERENCES BETWEEN TIMES OF REDUCTION OF INOCULATED AND UNINOCULATED TUBES ARE NEGLECTED. THE ARROW INDICATES THE PRESUMED Eh OF GERMINATION. (VERY APPROXIMATE)

1. Eh.

The effect of this factor is evident from Fig. 5. The greater the reducing intensity of the medium the more rapid the germination. This fact, however, can be shown more strikingly by using the same medium under the same conditions, but in one case inducing a negative Eh with $Na_2S_2O_4$.

Experiment 5.—Take 14 tubes ordinary broth; heat and cool as before. Adjust all to pH 8·1* with N/5 soda (amount previously determined). Tint all with indigo 3 and inoculate. Divide into two equal groups, and to one add enough (previously determined) $Na_2S_2O_4$ to cause immediate reduction (Appendix VII, p. 173). Cover all with ½ inch of vaseline. Set vaseline in water, and then mix by inverting and warm to 38° C. approximately. Incubate and note the time. Open and examine a $Na_2S_2O_4$ tube in 2 hours and subsequently every half hour. Treat the other tubes similarly every hour after 14 hours, the experiment being started at a time to make this possible (or the 2 series being started at different times). Germination in the $Na_2S_2O_4$ tubes will be detected after 2½–4 hours' incubation, in the others after 15–20 hours. The Eh of the $Na_2S_2O_4$ tubes will be on the negative side of −0·2 immediately and of the others, as judged by the dye, falling from about + 0·2 to about − 0·15 (Appendix VIII, p. 173).

2. Nutritional Factor.

It is obvious that even though Eh and pH be optimal the "nutritional" composition of the medium must effect lag. The spores will not germinate in

*pH 8·1 is selected, because if the medium is used at pH 7·6 germination will hardly take place at all (*cf.* Appendix V, p. 171)

a buffer solution at pH 7·6 containing $Na_2S_2O_4$. Neither will germination take place in peptone water so rapidly as in broth, even though $Na_2S_2O_4$ be added to each.

Experiment 6.—The lag of *B. tetani* in peptone + $Na_2S_2O_4$ was determined as in Experiment 5 under vaseline. The lag times are more variable than with broth but are usually about 14 hours. This time compares with about 3 hours for broth + $Na_2S_2O_4$ under vaseline. Thus even when the reducing intensity is great ($Na_2S_2O_4$), the addition of meat infusion shortens the lag time from about 14 hours to about 3 hours.

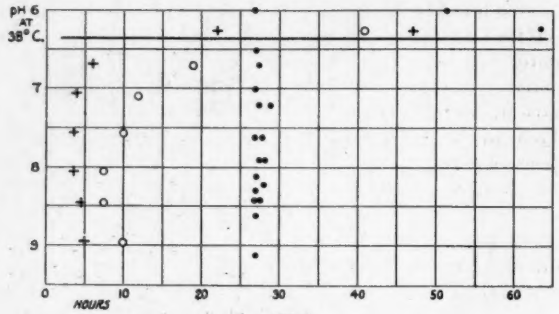


FIG. 6.
LAG TIME IN DIFFERENT MEDIA AT VARYING pH.
+ = ORDINARY BROTH + $Na_2S_2O_4$.
O = ORDINARY BROTH.
• = 10 PER CENT. ORDINARY BROTH IN PEPTONE WATER.
THE OBSERVATIONS ABOVE THE HORIZONTAL LINE AT pH 6·35 SHOWED NO GERMINATION; BELOW THIS LINE GERMINATION.

3. pH.

When the optimum pH for germination of *B. tetani* is determined in the usual way, *i. e.* growth in broth at different pH and occasional sampling, it is found with great regularity to be 7·6–7·8, *i. e.* germination starts first at this pH. In such an experiment, however, the result is a compound of pH and Eh effects, and to arrive at the real pH effect the Eh effect must be reduced to a minimum.

Experiment 7.—The lag time was determined in 3 media of different reducing intensities at different pH.

The 3 media were ordinary broth containing sufficient $Na_2S_2O_4$ to reduce indigo 4 immediately; ordinary broth and peptone water containing 10 per cent. broth. The techniques of heating, cooling and adjusting pH, etc., were as before. Each medium was dealt with separately, 6 tubes having a graduated pH being put up in each of 5 anaerobic jars. The pH of the medium was determined at about 38° C, not at room temperature; this gives a reading about 0·15 pH on the acid side of that at room temperature. The jars were opened at a time when previous experience suggested that germination was likely to have taken place.

Fig. 6 shows the times at which germination was observed at each pH.

This experiment indicates that in any medium the limiting pH on the acid side is about pH 6·35 (38° C.), or 6·5 at room temperature. On the alkaline

side the limit was not determined. In a medium of high reducing intensity the optimum (shortest lag) was in the neighbourhood of pH 7·8, whereas in a medium of lower reducing intensity (broth) it was more on the alkaline side (pH 8·2). This is unusually high. In the case of peptone water the curious result was obtained (repeated on three occasions) that no optimum was demonstrable. The figure also shows that in a medium of high reducing intensity pH has much less effect upon lag than in broth, whereas in peptone water of low reducing intensity pH has no effect at all within the whole range in which germination can take place. In the case of broth + $Na_2S_2O_4$ and broth alone the result is according to expectation. In broth pH has a marked effect on drift, and therefore it must have a marked effect upon early attainment of a negative Eh. In broth + $Na_2S_2O_4$ this pH effect on Eh is almost masked by the much greater effect of $Na_2S_2O_4$ on Eh. The effect of peptone water is less easy to explain. It seems, however, most probable that the effect of pH, which in the other two cases clearly operates through Eh, is not visible in this medium, because it largely lacks the reducing groups upon which the pH effect is exerted.

However, the outstanding conclusion which may be drawn from this experiment is that pH has very little direct influence upon rapidity of growth as far as pH 6·3 (38° C.) on the acid side. Beyond this limit, however, growth is totally inhibited (Appendix IX, p. 174).

It may now be concluded that if a medium of low reducing intensity at low pH is used for determining the most positive germination point, the low pH will not directly interfere with rapid germination. The low reducing intensity, however, when obtained by reducing the quantity of meat infusion in the medium, will cause a delay in germination for "nutritional" reasons. It will, however, be found that this delay is not so great as the delay in negative drift, and therefore on the balance it is advantageous to use a medium of this sort.

DETERMINATION OF THE POSITIVE LIMIT OF Eh IN A MEDIUM OF SLIGHT REDUCING INTENSITY.

The positive limit of germination was now tested under the conditions determined in previous sections.

Experiment 8.—Applicable technique as before. Medium, peptone (1%) water containing 10% ordinary broth; pH adjusted after heating and cooling with N/5 acid and alkali; thionin as indicator; 7 tubes arranged in front of a white card in the anaerobic jar; 5 separate sets put up; 1 jar opened from time to time.

Result at 27½ hours:

pH (38° C.).	Reduction of thionin.	Germination.	Eh.
8·9	100%	Starting	− 0·05 } or more
8·4	100%	,,	− 0·03 } negative.
7·9	90%	,,	+ 0·01
7·6	50%	None	+ 0·05
7·2	80%	Starting	+ 0·04
6·7	70%	,,	+ 0·06
6·3	30%	None	+ 0·09

The degree of reduction of thionin was judged before opening the jar by a scale made by dilution (with the medium) of a tube of medium tinted to the same degree as the test-tubes. This scale must be made at the time of reading; if made when the test is put up and kept in air it will fade somewhat.

This experiment shows that at the alkaline end of the pH series germination took place at an Eh approximating to that of 98% reduction of indophenol 1, whereas at the acid end the Eh of germination was more negative.

This result is approximately constant with the same technique.

Result at 27 hours:

pH (38° C.).	Reduction of thionin.	Germination.	Eh.	Next day.
9·0	100%	Starting	− 0·06	Visible growth
8·6	90%	,,	− 0·01	,,
8·0	80%	,,	+ 0·01	,,
7·6	70%	,,	+ 0·03	,,
7·0	60%	,,	+ 0·06	,,
6·5	50%	,,	+ 0·08	,,
6·0	0	None	...	No germination.

In this case the positive limit is somewhat higher. At the alkaline end the Eh is that of 80% reduced indophenol 1, but at the acid end it is below the 98% reduction point of this dye.

Criticism of Previous Methods of Determination.

The methods which have been described so far have presumed that the behaviour of the dye in the tube of medium is a sure indication that the medium as a whole stands at the Eh appropriate to the state of the dye, and that spores suspended in the medium are subject to the Eh so indicated.

It is not, however, certain that this is the case. It will be understood that a tube of medium exposed to the atmosphere acquires its reducing intensity by virtue of the reducing properties of its constituents working in opposition to a constant oxidation from the top by atmospheric oxygen. When, therefore, the medium is at rest in a test-tube exposed at the open end to the atmosphere, the reducing intensity will be most effective and persistent at the bottom of the tube, and this region will develop a more negative reduction potential than the top. This can readily be demonstrated by boiling out a tube of glucose broth, cooling and tinting with indigo 4. If this tube is placed into the incubator, and examined without introducing convection currents by chilling, the whole tube except the top centimetre will be found reduced in 1 hour, and the area of reduction will shrink until in 24 hours the bottom $\frac{1}{2}$ inch of the medium will be found yellow and the rest blue.

Even when a tube is not open to the air but is closed with vaseline, the oxygen dissolved in the vaseline is sufficient to exert a continual oxidizing action at the surface, so that a tube of glucose broth tinted with methylene blue for instance and covered with vaseline will show, at about the time of complete reduction, streams of blue continually falling from the vaseline.

With such an arrangement, therefore, the bottom of the tube is apt to have a higher reducing intensity than the top.

On the other hand, when a medium with a slight content of O_2 is enclosed in a test-tube and placed in the anaerobic jar, the surface will develop a higher reducing intensity than the depth, owing presumably to diffusion of the gas from the surface and its combustion by the platinum-asbestos. This may be shown by heating ordinary broth to 100° C. for 2 minutes, in order to expel gross air from solution without developing a high reducing intensity. This broth is tinted with indigo 4 and filled into a test-tube shaped as in Fig. 7. This tube is then put up in the anaerobic jar at 37° C. Next day the body of the tube is blue and the superficial part of the fluid yellow. Hence the surface of a medium of low reducing intensity under good "anaerobic conditions" may develop a greater reducing intensity than the depths.

FIG. 7.

Many further illustrations of these effects might be brought forward to show that different parts of the same tube of medium may at the same time show different reducing intensities.

In the examples given the regional differences in Eh are so gross that they are easily detected by the alteration in the dye, but it is obvious that small differences in Eh may also exist which cannot be detected by alteration of colour. If, for instance, there was a mere surface reduction this would not be visible, and a spore occupying this position and brought to germination by the conditions in it would be presumed to have germinated at the Eh indicated by the whole mass of dye. Thus the presumed Eh of germination may be more positive than it actually was.

In addition to a patchy distribution of Eh due to the medium itself, there is a probability of a similar effect from the inoculum.

In Appendix VI (p. 171) are given reasons for supposing that a suspension of spores may elaborate reducing substances before germination takes place. Now if the spore is so situated in the test-tube that these reducing substances are not removed by diffusion, it is easy to see that the local Eh may become negative more rapidly than it does in the rest of the medium, and so apparently permit germination at the more positive point indicated by the general mass of dye. Spores deposited on the bottom of the tube or in a particle or clump will be liable to behave in this manner.

Possibilities of this sort made it necessary to compare previous results with data obtained from tubes in which the contents were kept homogeneously mixed, so that the state of the dye might certainly indicate the conditions to which all spores were subjected.

The method adopted was to bubble hydrogen through the cultures.

This technique had also the advantage of offering a method by which the objection might be overcome that the spores in previous experiments were suspended in a medium of poor "nutritional" value, necessitated by the requirement of low reducing intensity. In the hydrogen bubbling method it was possible to use a medium of greater reducing intensity and nutritional

value, namely ordinary broth, and hold this at a positive Eh for a long time by means of traces of air in the hydrogen.

Further, an opportunity was afforded to measure the Eh by addition of reduced dye at the end of the experiment. Thus any possible effect of the dye in the medium could be avoided.

DETERMINATION OF THE POSITIVE LIMIT OF Eh IN AN AGITATED MEDIUM.

The technique adopted in these experiments was as follows:

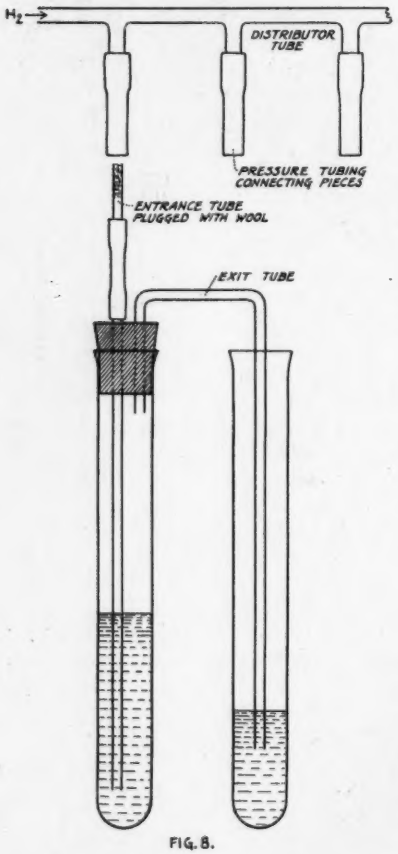


FIG. 8.

The tubes of broth, heated, cooled, adjusted and inoculated as before were fitted with rubber corks and tubes as shown in Fig. 8. No indicator was added. The apparatus was placed in a rack with the exit tube leading into an empty test-tube. The entrance tube was then pushed into the connection of the distributor tube, and the whole attached to the hydrogen supply to the incubator by means of a tube long enough to allow the rack to be removed from the incubator for inspection. Water was then added to the empty test-tubes to cover the orifices of exit to about $\frac{1}{2}$ inch and the hydrogen turned on. The amount of water in the water tubes was then adjusted so that the rate of bubbling in each tube at lowest pressure was as nearly equal as possible. Place in the incubator and start passage of hydrogen.

The most convenient way for passing hydrogen through the tubes was found in the apparatus shown in Fig. 9.

By means of the constant dripping bottle, water was passed at about 30 drops per minute into a large test-tube which siphoned over 50 c.c. about every 15–20 minutes and discharged into the gas tank. The sudden rise of head in the narrow side arm caused a sudden discharge of gas through the tubes of medium, and was able to overcome any slight differences in resistance between the various tubes. The quantities stated here are satisfactory for 12 tubes of broth.

The manipulation of the tank is described in Appendix X (p. 174).

Owing to unavoidable irregularities in the rate of bubbling in each tube, it

is difficult to give precise directions for maintaining the cultures at a positive Eh. When the pH is about 6·7 the reducing intensity of the medium must be increased by adding about 10% glucose broth, and the amount of air in

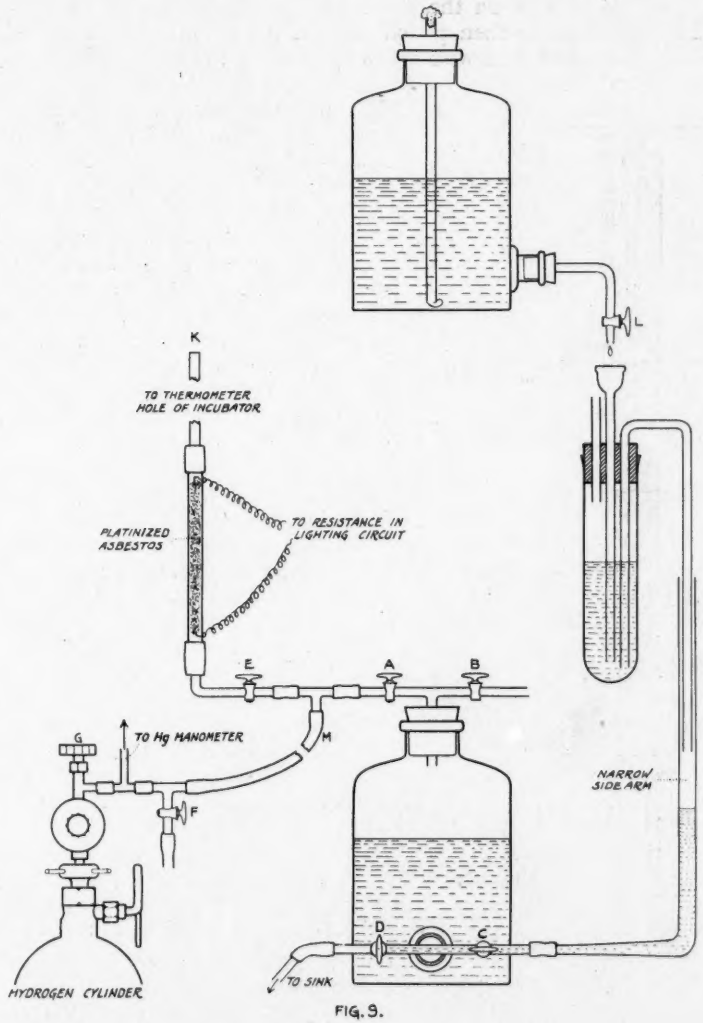


FIG. 9.

the hydrogen must be reduced as far as possible by passing it through the electrically heated platinized asbestos. On the other hand, when broth at pH 7·7 for instance is used, the heater is turned off and even air may be added to

the tank (about 2·5%). The idea of these measures is to keep the Eh of the tubes of medium on the positive side of 98% reduction of thionin for 24 to 48 hours.

From time to time a tube is removed for examination. This is achieved by putting a screw clip on the pressure tubing connection with the distributing tube (Fig. 8) and another on the short piece of tubing under the cotton-wool plug. The apparatus is then pulled off, but before putting on the clip it is better to cool the tube, otherwise the water sealing the exit tube may be drawn back into the culture.

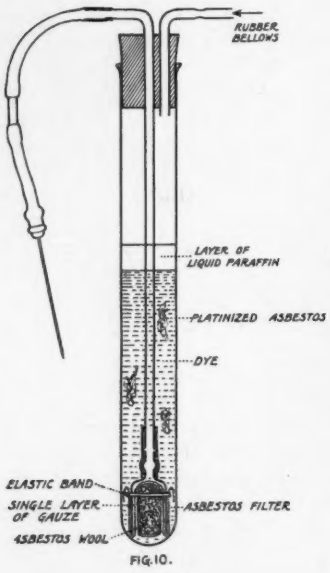


FIG. 10.

It is now neecessary to inject rduced dye into the culture without interfering with the oxidation-reduction balance. This was done as follows:

An apparatus was constructed as in Fig. 10. The tube was half filled with a solution of dye in saline (not water), of such a strength that about 1 c.c. added to a tube of broth would impart a strong colour. A good pinch of platinized asbestos was added and shaken in the dye. The filter funnel was now inserted below the level of the dye and ½ inch liquid paraffin poured in. The cork was at once wedged in and hydrogen passed through. This was done most conveniently by attaching to F (Fig. 9) a piece of thin tubing blocked off at the distal end. The needle was pushed through this and the gas turned on (a clip being on at M, Fig. 9). The hydrogen was passed until the dye was entirely reduced.

It should be borne in mind that the asbestos filter in the apparatus must be tight enough to prevent any trace of platinum black passing, but loose enough to pass fluid at a reasonable pressure. The filter is put into the dye before the paraffin to prevent it from being clogged, and the hydrogen is passed without delay to prevent the filter being soaked with unreduced dye, which would not then come under the influence of the platinum black.

When the dye is reduced turn off the hydrogen at G, attach a rubber bellows and blow up the reduced dye nearly to the needle before shutting F, clipping the tube and removing the needle. Discard several drops, and then stab the needle through the rubber connection of the culture tube below the clip. Pump in about 1 c.c. and remove the needle; the positive pressure equalizes through the exit tube. Agitate the mixture of culture and reduced dye to make it homogeneous; allow to stand for 20 minutes and take a reading of the tint (*vide* below); then screw out the cork and remove it with any dye still in the entrance tube and take a sample for determination of germination. Shake the tube to re-oxidize it and read the tint again, finally adding a drop of potassium ferricyanide to make certain that the full colour has returned.

The reading of the tint is made by comparison with a graduated series made by dilution of the dye with broth (the series not stable). The strength of the dye must be such that the strongest concentration will include the tint of the oxidized test-tube. The rest of the series consists of known fractions of this to the limit of visibility. Thus by comparing the readings of the test-tube before and after oxidation it is possible roughly to estimate the degree of reduction before it was opened, and knowing the pH, to read the Eh from data already given.

An error may be introduced into the estimate of reduction by reason of the fact that with the more positive dyes a certain time is necessary for the development of the reduced tint. This tint, however, seems to become stable in 20 minutes. In cases where the hydrogen contains more than traces of oxygen, the gas over the culture will cause a progressive oxidation of the reduced dye and an estimate cannot be made. It is therefore always desirable to turn on the heater and pass gas as pure as possible through the culture for at least an hour before it is removed for examination. Even then with the apparatus in use a number of tubes are valueless for anything but a rough approximation.

In experiments of this sort it is a matter of chance whether germination is present or not, because the bubbling of hydrogen is too inaccurate to give a result which can be anticipated. A tube cannot be opened (as it can in the anaerobic jar) with confidence derived from previous knowledge that germination will be present.

Generally no germination is found, but occasionally there is heavy vegetation or visible growth. The Eh of the tubes is hovering on the border-line, and if it just reaches the effective limit and germination starts, it rapidly becomes negative. Such tubes are therefore useless for measurement, but serve as a control of the possibility of growth taking place. Those tubes which do not overstep the limit remain without germination, and therefore many of the results of the experiments have to be given in terms of Eh at which germination did not occur although time and other conditions were all favourable. Occasionally, however, a tube may be found in the act of germination, and this will then give direct evidence as to the Eh at which germination occurred.

Fig. 11 indicates the results obtained using indophenol 1 as indicator. Each dot shows a culture which had not germinated in 24 to 48 hours at the pH and Eh indicated. The vertical arrows show that cultures in which germination was observed were all at an Eh minus to 100% reduction of this dye.

Fig. 12 shows the results with thionin as indicator. Here again lack of germination was observed in all cases (except one) in which the Eh was positive to the 98% reduction line. At pH 6·8, 7·2 and 7·6 cultures were examined by chance at the moment of germination, and the Eh was found to coincide roughly with the reduction point of this dye.

The details of one experiment are given as a sample:

Experiment 9.—12 tubes containing broth plus 10% glucose broth; pH 7·2; heater on; dripping 30–35 drops per minute and tubes examined in 24 hours.

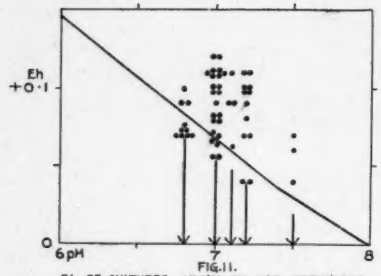


FIG. 11.
Eh OF CULTURES WHICH DID NOT GERMINATE AS COMPARED WITH Eh OF 98 PER CENT. REDUCTION OF INDOPHENOL 1.

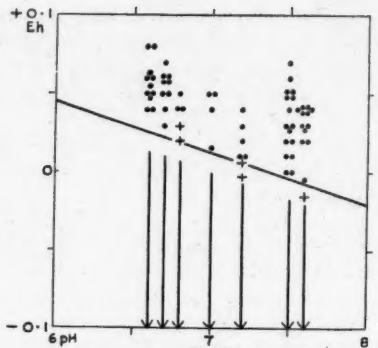


FIG. 12.
Eh OF CULTURES WHICH DID NOT GERMINATE (-) AND OF THOSE WHICH WERE JUST GERMINATING (+) AS COMPARED WITH Eh OF 98 PER CENT. REDUCTION OF THIONIN.

Tube	1 =	98% reduction	= Eh	+ 0·005	= blue and red spores.
,,	2 =	98% ,,	= ,,	+ 0·005	= ,, ,,
,,	3 .	. . . .	. .	.	visible growth.
,,	4 .	. . . .	. .	.	,,
,,	5 =	98% ,,	= ,,	+ 0·005	= one spore germinating, others blue and red.
,,	6 =	90% ,,	= ,,	+ 0·03	= red spores.
,,	7 =	100% ,,	= ,,	− 0·005	= early vegetation.
,,	8 =	70% ,,	= ,,	+ 0·04	= red spores.
,,	9 =	95% ,,	= ,,	+ 0·02	= ,,
,,	10 =	100% (practically) ,,	= ,,	− 0·005	= one spore germinating, others blue and red.
,,	11 =	100% reduction	= ,,	− 0·005	= active vegetation.
,,	12 .	. . . .	. .	.	visible growth.

Tubes 3, 4 and 12 are represented in Fig. 12 by the arrow. Tubes 7 and 11 are also represented by the arrow, because the vegetation had been present long enough to affect the Eh. Tubes 5 and 10 are shown as crosses and the remaining 5 tubes as dots, although tubes 1 and 2 had obviously started the process of germination.

The conclusion to be drawn is that germination starts approximately at the Eh of reduced thionin; and that germination failed in some cultures because their Eh was not negative enough.

The second method adopted for measuring the Eh for germination has thus resulted in evidence that the true Eh necessary before the spores will germinate is more negative than the former method indicated. This is in accordance with expectation. By the former method at pH 7·0 the point was determined as Eh + 0·05 to + 0·06, *i. e.* just negative to the reduction point of indophenol 1, whereas the second method indicated about + 0·01 in the neighbourhood of the reduction point of thionin. For the reasons advanced it is supposed that the more negative point is more likely to be correct.

CONCLUSIONS.

1. On exclusion of air, culture media develop a reducing intensity which can be measured by Clark's indicators of oxidation-reduction potential.

2. The rapidity and extent of this change depends upon the constitution of the medium and upon pH.

3. The period required for the germination of spores of *B. tetani* (lag) in a suitable medium at 38° C. depends mainly on the time required for this medium to reach a suitable reducing intensity.

4. Within limits, pH does not affect the lag of *B. tetani* except through its effect upon the reducing intensity of the medium.

5. The greater the reducing intensity of the medium the shorter the lag and, conversely, the slighter the reducing intensity the longer the lag, until a zone of reducing intensity is reached at which germination is not observed to occur at all.

6. This zone may be measured and expressed in terms of oxidation-reduction potential (Eh). At each pH it approximates to the zone of complete reduction of thionin—*i. e.* at pH 7·0 it approximates to Eh + 0·01 volts.

7. If spores are placed under conditions in which the Eh is more positive than this, they do not germinate.

I wish to record my indebtedness to Mr. R. K. Cannan, Dr. H. D. Kay and Dr. P. P. Laidlaw for valuable advice during the progress of this work.

Appendix I.—Preparation of the Dyes.

Thionin and the more negative indicators can be sterilized in the autoclave, but the indophenols are easily decomposed by heat.

The indophenols and thionin, in particular, are not permanently stable in watery solution. They should be freshly prepared at least every week, and kept in the refrigerator.

The lack of stability of the dyes does not necessarily lead to any conspicuous change while in watery solution, but when added to a medium the colours may be found feeble and subject to rapid fading. This fading is not to be confused with reduction. Owing to possibility of error it is always necessary to check the fact of true reduction by showing that the full colour will return on shaking with air, or in the case of the more positive indicators, by adding potassium ferricyanide and shaking.

Although the indophenols cannot be sterilized by heat, sterile solutions may be readily prepared simply by dissolving the dye in sterile water with the usual precautions.

Since small quantities only are required to be made repeatedly and weighing under sterile conditions is difficult, the solutions used have not been made to any particular strength. The strength has been such that 1, 2 or 3 drops (1 drop = about 0·03 c.c.) added to 10 c.c. of medium in a $6 \times \frac{5}{8}$ in. tube have imparted a minimal convenient tint to the fluid. When it is required roughly to estimate degrees of reduction by comparing a tube with a

similar tube diluted to various degrees with broth, more dye than the minimal quantity must of course be added.

The samples of dyes used were obtained from British Drug Houses, Ltd., Graham Street, City Road, London, N. 1.

Appendix II.—The Inoculum.

The suspension of spores used in the inoculation should be made from a strain which autolyses easily and spores well in order that no difficulty may arise in judging germination. The spores consisted of a surface growth, washed and heated to 65° C. for 1½ hours. Some of the suspensions used were fresh and others three years old. The suspension was diluted with saline to such an extent that when 1 drop was added to 10 c.c. of medium and 1 drop of this mixture examined without spreading, 1 spore was found in about 2 or 3 fields.

The spore suspension was prepared in this way in order that the inoculum might contain a minimum of the products of previous growth.

Appendix III.—The Colours of the Dyes.

In the oxidized state the indigo dyes are blue and bright yellow when reduced, indigo 4 being the brightest. The colours of methylene blue and thionin are familiar; they are colourless when reduced.

The indophenols as a class are unfortunately subject not only to the change of colour due to Eh but a change due to pH. In watery solution they are all blue in alkali and red in acid; in both cases when reduced they are more or less colourless.

The relatively simple indophenols, o-chlorphenol, o-cresol, 1 naphthol–2 sulphonate are not blue within the limits of pH 6·0 to pH 8·0, and therefore when dissolved in broth show a blend of yellow-brown with a tinge of dirty blue. Under these circumstances it is extremely difficult to judge when complete reduction has taken place and impossible to judge any degree of reduction. They therefore could not be used in this work. This fact reduces the number of satisfactory dyes available in a most important zone to 2, namely, phenol-indo 2·6 dibromophenol and 1 naphthol–2 sulphonate indo 2–6 dibromophenol. These are blue within the pH limits mentioned and more or less colourless on reduction.

Appendix IV.—Germination.

The germination time is arrived at as follows:

The tube is opened, a sample removed with a pipette and 1 drop placed on a slide without spreading. This is stained in the usual way for spores with carbol-fuchsin and methylene blue. On examining a culture in which the reducing intensity is great, the following changes are seen at about 30-minute intervals:

1. Red spores become intense blue and elongated.
2. Spore-case ruptures and intense blue bacillus—very broad and short—is usually seen half "hatched."
3. Intense blue bacillus—still broad—elongates outside the spore-case, and subsequently starts dividing in the sualu way.

A freshly germinated bacillus is conspicuously different from a ghost of the last generation by the intensity of its staining and its width.

When the reducing intensity of the medium is slight the whole process becomes so long that any particular stage of germination is not likely to be noticed. It is then a question of red spores or early external vegetation.

It will be recognized that the determination of the germination time must be made with some accuracy, since when germination becomes active a rapid increase of reducing intensity takes place. If the observation is much delayed, the Eh of the culture may be more negative than it was at the time of germination.

Appendix V.—The Sequence of Reduction.

When the series of dyes is put up in a medium such as broth and then subjected to anaerobic conditions, it would be expected that the most positive indicator should be reduced first and the others in their proper order, as is shown in Experiment 1. This, however, does not happen unless only very small quantities of dyes are added, because some dyes "poise" the system in the oxidized range and thus delay the drift to their own reduction point.

The expression "poising" is used by Clark and his collaborators to denote an effect in Eh which is akin to "buffering" in pH. All dyes poise in the neighbourhood of 50% reduction, *i.e.* they take longer to pass through this zone of Eh than through the 0–25% reduction and the 75 to 100% reduction zones. But some dyes, whether owing to impurities or not, when used in the manner described, certainly delay the drift to a greater extent than this, especially when the reducing intensity of the medium is slight.

Methylene blue is specially prone to act in this manner, but the two indophenols also behave similarly. The effect becomes more pronounced as the concentration of the dye increases.

If the poising dyes are capable of restraining the negative drift of a medium, other dyes have the opposite effect. Thionin, for instance, becomes reduced with undue facility; it accelerates the negative drift.

These anomalies in the behaviour of indicators are not as yet capable of theoretical explanation. For the present, therefore, interpretation of indicator studies may be properly based upon the general trend of the mass of data, obvious abnormalities being neglected.

These characters of the dyes have an effect upon germination which must be borne in mind. For instance growth will take place in a tube of medium containing thionin when no growth will occur without thionin. This is conspicuously the case when "anaerobic conditions are bad"—for instance when vaseline is used to exclude atmospheric air. The vaseline occludes and imparts so much oxygen to the culture that there is a continual positive bias against the negative drift of the medium, with the result that when ordinary broth is used, the Eh (at pH 7·5) never falls low enough for germination. When thionin is present, however, sufficient drift does occur. Thus growth takes place in ordinary broth and thionin under vaseline, but not without thionin.

Appendix VI.

Hitherto in this paper it has been assumed to be common knowledge that when a medium is tinted with dyes and placed under anaerobic conditions,

reduction of some will take place, *i.e.* that it will develop an Eh more negative than at the start. Quastel and Stephenson (1926), however, when cultivating *B. sporogenes* in Cole and Onslow's tryptic broth, observed that growth did not occur until 66 hours had elapsed. They pointed out that during the long delay "either the inoculant or the medium has changed towards a condition favourable to anaerobic growth," and concluded that the change was in the inoculant, because "it is unlikely that tryptic broth alone will alter its composition during anaerobic incubation at 37° C." They went on to advance the hypothesis that the bacteria inoculated, although inhibited ("resting") and unable to grow, were digesting the sulphur compounds of the medium and producing sulphur groups which were responsible for lowering the potential of the medium.

I have no knowledge of the behaviour of Cole and Onslow's medium, but a similar phenomenon may be observed in peptone water, namely germination after long delay. In the case of peptone water, however, it may be shown that the medium itself does change towards a condition favourable to anaerobic growth. Thionin becomes reduced in about 3 days at pH 7·6.

When two dye series are put up, one inoculated and the other not, there is seldom to be observed a more rapid drift in the inoculated series, and then only when the medium is of slight reducing intensity. Thus in an experiment with peptone water + 10% of broth (pH 7·6), indophenol 1 was reduced in 22 hours in the inoculated series but only 60% reduced in 26 hours in the uninoculated. However, in a third series inoculated with spores which had been washed by centrifuging, the reduction-rate was exactly the same as in the uninoculated series.

It will be noted that the spores used were prepared in such a way as to contain little of the original culture (Appendix 2), but Quastel and Stephenson used as an inoculation 2 or 3 drops of an 18-hour tryptic broth culture in a total bulk of 5 c.c. of medium. The dilution of their inoculum might, therefore, have been only 1 in 33, and it would consist not only of living vegetative forms, but also of the sulphur groups responsible for reduction. Thus on the evidence supplied in their paper it would seem possible that the change in the medium, if not due to a spontaneous drift, might be due to these preformed products of culture rather than to the activities of "resting" bacteria.

In my experiments, which were not designed for the purpose, I have found no evidence of an actual production of reducing substances by a suspension of spores in the sense that a reducing activity has been demonstrated to have developed which was not present before. On the other hand, I have observed the production of toxin by a suspension of spores. On two occasions suspensions of spores after going through the usual process of heating (and also carbolizing) have of course been non-toxic, but after remaining at −2 or −3° C. for about two years they have become toxic (local tetanus in the guinea-pig). In this case I have assumed that the dead bacilli have contained enzymes, not entirely destroyed at 65° C., which have slowly continued their normal functions in the same way that dead staphylococci continue to respire. This assumption would seem reasonable in view of later publications by Quastel and his collaborators (Quastel, 1928).

Appendix VII.—Sodium Hydrosulphate.

$Na_2S_2O_4$ is a very convenient reagent for inducing high reducing intensities in media. It forms an approximately neutral solution in water and decomposes on heating. It cannot, therefore, be sterilized by heat. Since fresh solutions are always used and they must be prepared aseptically, it is most convenient to make them up without weighing, but approximately 1% with a sterile spatula. When it is desired to induce intensities of reduction greater than that at which a certain dye reduces, the solution is added to a tube of medium containing this dye until reduction takes place. This quantity can then be used in other tubes.

Appendix VIII.—Effect of the Strain of Spores on Lag.

Up to now no mention has been made of the strain of spores used. It seems clear that under constant conditions the lags of all strains are not the same. Some grow rapidly and others more slowly. In an effort to obtain germination at the most positive Eh, *i.e.* as early as possible, a rapidly germinating strain must be used.

When 14 strains (spore suspensions) were put up in broth with $Na_2S_2O_4$ under vaseline the following lags were observed:

			Lag.	Visible growth next day.
Tulloch	type	1	3 hours	+
,,	,,	2	3 ,,	+
,,	,,	3	3½ ,,	+
,,	,,	4	3 ,,	+
,,	,,	5	3 ,,	+
Tenbroeck	,,	7	3 ,,	+
Fildes	T.M	8	>6 ,,	+
,,	,,	10	>6 ,,	+
,,	,,	11	3 ,,	+
,,	,,	13	3 ,,	+
,,	,,	14	3 ,,	+
,,	,,	15	>6 ,,	+
,,	,,	16	3 ,,	+
,,	,,	17	5 ,,	+

Thus 4 of these strains showed a definitely longer lag than the others, and these 4 were among recently isolated strains from man. The explanation of this longer lag may be twofold:

First, these recent strains may require longer to adapt themselves to the abnormal nutritional surroundings of the culture medium.

Secondly, they may be inhibited a little by an inadequate Eh, *i. e.* they may be more "anaerobic."

It seems improbable that the reducing intensity can be inadequate, but nevertheless the following experiment, at first sight, seems to support this idea:

The 14 strains were put up in glucose broth which had been boiled for 10 minutes and placed into cold water for 10 minutes. Care was taken not to shake the tubes. They were placed in the incubator under aerobic conditions

and subsequently observed only through the glass door, all this being to avoid mixing the contents by convection currents or otherwise. At the same time an uninoculated tube was prepared with a tint of indigo 4.

In ¾ hour the tube of indigo 4 was reduced except for the top centimetre. It was in the same condition in 5½ hours. Next morning all strains showed a visible growth in the aerobic glucose tubes except the same 4 in which the lag was long under good Eh conditions. A phenomenon of this sort would immediately lead to the conclusion that these 4 were more anaerobic than the others, but it is doubtful whether this would be correct.

Examination of the 4 tubes showed that germination had taken place, but the growth never became visible in 3 days. The indigo 4 tube, which was almost completely reduced during the first few hours, was completely oxidized next morning.

It seems, therefore, that the true explanation is as follows: The bottom part of each tube was "anaerobic" for at least 5½ hours. During this time the short lag strains germinated, and by the activity of their growth maintained a satisfactory Eh against the oxidizing action of the air. The long lag strains just succeeded in germinating, probably only at the bottom of the tube, but had not had time to produce enough reducing substances to withstand the wave of oxidation falling from the top of the tube. Their failure to grow continuously was therefore due to their slowness in germinating, not to more stringent anaerobic requirements.

Unless otherwise stated all tests in this work have been made with a quick germinating strain—Tulloch's type V.

Appendix IX.—Limiting pH of Other Strains.

This acid limit given here is considerably more alkaline than the limits given in the literature and therefore it was tested on 12 different strains.

Strains used:

Tulloch's U.S.A.; T 67; R 220; Type V.
Tenbroeck's Type VII.
7 recent strain from man.

All made up as usual as spores; broth pH 6·2 (38° C.); anaerobe jar.

Result: 3 days—No visible growth.
4 days—Visible growth in T 67 and 2 recent strains. No visible growth (or germination) in the others.

This experiment suggests that all strains of *B. tetani* have approximately the same difficulty in germinating at or about pH 6·2–6·3 (38° C.).

Appendix X.—The Gas Tank.

It will be seen (Fig. 9) that the tank is filled as follows:

1. Shut taps A and C; open B and D.
2. Attach D to water supply and fill the tank. Shut D.
3. Shut E, F; open A.
4. Blow hydrogen through by operating tap G on the reducing valve. Endeavour to remove air from the dead space at the top of the tank by occluding B with the finger and then allowing the gas to burst out.

5. Shut B and open D. The tank will then fill with gas.
6. Force water into the narrow side arm by shutting D and opening C.
7. Open D again and adjust the flow at G so that the level in the narrow side arm is constant.
8. When the tank is full, shut G and D. When the row of tubes is attached to K, shut A and open E; blow through the gas by adjusting G. When bubbling is to be started, adjust L, and when the siphon discharges open A and shut G. It is better to put a clip on M while the experiment is in progress.

If the tank is calibrated, roughly measured proportions of air may be drawn in during filling.

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Note.—Added April 4th, 1929. I have just read a paper by C. B. Coulter, *J. Gen. Physiol.*, 1928, **12**, 139, and another by R. Dubos, *J. Exp. Med.*, 1929, **49**, 507. Both deal with the negative drift of Eh in sterile broth. My results, so far as they apply, seem to be in general agreement.

SEASONAL VARIATION IN THE SUSCEPTIBILITY OF MICE TO DYSENTERY (SHIGA) TOXIN.

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Received for publication March 3rd, 1929.

MOST investigators engaged in the standardization of toxins, sera or drugs on animals have probably been conscious that animal populations vary from time to time in their susceptibility to the particular effect studied. Not only is variation of susceptibility among the individual animals always apparent if sufficiently fine measurements are made, but on different occasions the whole group may show a variation in susceptibility. This is evidenced by a definite move in one or other direction of the "frequency distribution" expressing the results of the experiment, or in other words there is a new mean to the observations, and the individual variations from it are proportionally the same on the two occasions. An apparent change in susceptibility in one isolated experiment must not be ascribed to factors affecting the animals alone, since most biological reagents are liable to deterioration, and such deterioration may produce the same effect as variations in the susceptibility of the animals.

For example when determining the L+ dose for a toxin by injection of

toxin-antitoxin mixtures the same mixture may give different lethal effects on two or more occasions; more than the expected number of animals may survive or more than the expected number may die. In the former case one must consider the possibility of the deterioration of the toxin and in the latter of the antitoxin. When no collateral evidence can be found that the reagents have deteriorated, if the variation takes place first in one direction and then in the other, and above all if the variations show a periodic distribution, one may ascribe it to factors affecting the animal group rather than the toxin and antitoxin.

The fact that there are in medical literature few records of group variations in susceptibility probably depends on two factors—the variations are only likely to be noticed in estimations where large changes in dosage produce relatively small changes in the effect studied, and their periodicity can only be established after many months' study and by the use of a large number of animals. The phenomenon is, however, an important one, and one seems likely to meet with it where any method of biological assay is carried to such a degree of accuracy that marked deviations from the mean result occur.

Reid, Hunt and Seidell (1910) working with acetonitril on mice found a variation in susceptibility to this drug which they considered to be due partly to the season and partly to the age and diet of the mice.

Trevan and Boock (1925) in their study of the standardization of insulin by the determination of the convulsive dose for mice, noted that the mean convulsive dose varied in groups of mice on different occasions though they were not able to correlate this variation with any particular factor.

Sudmersen and Glenny (1909) showed that it required a larger dose of diphtheria toxin to kill a guinea-pig of a given weight in summer than in winter. In the case of diphtheria antitoxin estimations the test dose of toxin contains so many lethal doses that the variability does not seriously affect the results of any titration which aims at the degree of accuracy usually demanded in such investigations. If, however, one had to titrate diphtheria antitoxin against a test dose of toxin containing very few lethal doses, then this phenomenon would have to be taken into consideration.

In the case of estimations of dysentery (Shiga) toxin and antitoxin the case is different. In order to kill 100% of mice it usually required as much as four times the dose of dysentery toxin required to kill 50%. Moreover, in an attempt to approach the accuracy of titration obtained with diphtheria toxin and antitoxin, the rough methods available for the titration of dysentery toxin and antitoxin have been strained to the utmost.

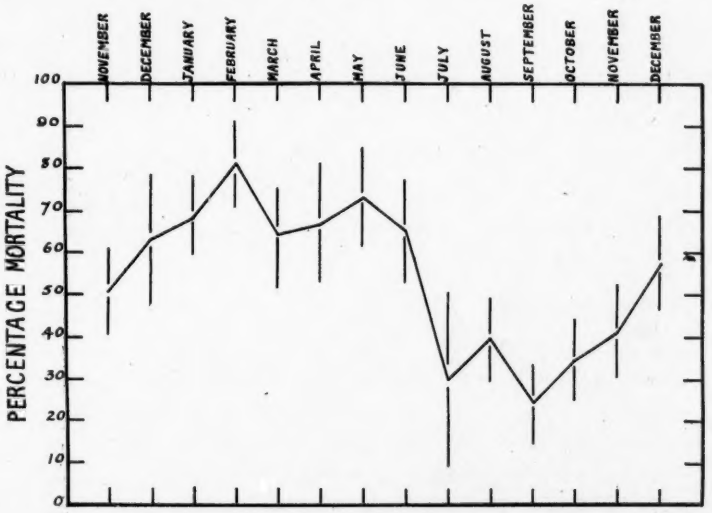
Many investigators, including Doerr (1924), have recorded the great variability in individual susceptibility of mice to dysentery toxin. In addition to this well-recognized individual variation, we thought it of interest to record a variability in groups of mice which we believe to be of a seasonal nature.

For several years we have used in this laboratory a test toxin 3A prepared by O'Brien and Runge (1925) consisting of the dried intact bodies of the Shiga bacillus. In the summer of 1926 it was found that the test dose* of toxin 3A

* A test dose of toxin as defined by the League of Nations Sub-committee is the amount of toxin which when mixed with 1 unit of Copenhagen standard serum (*i. e.* 0·005 c.c.) and injected intravenously into a group of mice will kill 33%, and with ⅔ unit of standard serum (*i. e.* 0·0033 c.c.) will kill 66% of mice.

(0·12 mgm.) when mixed with 1 unit of Copenhagen standard serum did not give the expected mortality of 33%. A possible explanation of this was that the test toxin had deteriorated, and to exclude this possibility standard test toxins from the State Serum Institute, Copenhagen, were then used in place of 3A. Again the mortality figure was consistently lower than 33%. This continued for several months, when unexpectedly in October a test dose of the standard toxin 3A and 1 unit of Copenhagen standard serum gave the normal mortality of 33%.

With a view to examining this variation in detail, it was decided to arrange an experiment where a constant toxin serum mixture was injected into groups of 20 mice at frequent intervals between November, 1926, and December, 1927. The same toxin 3A has been used throughout as test toxin, and the standard sera (DS I, DS II and DS III) as issued from the National Institute of Medical Research, London, and all these samples of serum are identical with the Copenhagen standard serum. The amount of toxin used was 0·12 mgm. and the amount of serum 0·004 c.c.



From the neutralization curve for dysentery toxin and serum (Trevan, 1929) this amount of standard serum ought to protect 50% in a group of mice.

The same technique has been used and the injections have all been made by one operator. The toxin and serum mixtures were incubated at 37° C. for 30 mins. before injection. All the mice used weighed from 16–18 gm. and were obtained from one carefully chosen source of supply. At different times during the course of the experiment samples of mice from other sources were used in parallel, and they always showed a susceptibility similar to the regular mice used at the same time. It would appear, then, that fluctuations in the susceptibility of mouse populations to dysentery toxin are to be expected whatever the source of supply of the mice.

The average mortality rate has been calculated for each month and plotted on a curve (Chart). It will be seen that the variability appears to be of a

TABLE I.

Date.	Number dead. Number injected.	Monthly percentage mortality.
1927.		
Nov. 1	8/20	
„ 15	10/20	
„ 15	8/18	50·6 ± 5·075*
„ 22	9/20	
„ 29	14/19	
Dec. 6	12/19	63·0 ± 7·83
„ 16	12/19	
1928.		
Jan. 5	14/20	
„ 11	15/20	68·8 ± 4·63
„ 17	13/20	
„ 24	13/20	
Feb. 1	18/20	
„ 8	14/18	80·7 ± 5·2
„ 14	14/19	
Mar. 12	16/20	
„ 20	10/20	64·3 ± 6·23
„ 27	12/19	
April 3	10/17	66·5 ± 7·87
„ 24	14/19	
May 2	15/20	
„ 9	16/20	73·3 ± 5·71
„ 31	13/20	
June 6	16/20	
„ 19	20/20	65·3 ± 6·2
„ 26	3/19	

Date.	Number dead. Number injected.	Monthly percentage mortality.
1928.		
July 24	6/20	30·0 ± 10·25
Aug. 1	8/20	
„ 9	10/20	
„ 14	7/18	39·8 ± 4·94
„ 21	7/20	
„ 28	7/20	
Sept. 4	2/20	
„ 10	6/20	24·2 ± 4·84
„ 18	4/20	
„ 25	7/19	
Oct. 2	7/18	
„ 9	6/18	
„ 16	8/20	34·4 ± 4·85
„ 23	8/20	
„ 31	4/20	
Nov. 6	9/20	
„ 13	9/18	41·25 ± 5·57
„ 20	4/20	
„ 27	10/20	
Dec. 4	12/16	
„ 11	10/20	57·0 ± 5·72
„ 18	13/19	
„ 31	7/20	

* The standard deviation calculated from the number of animals used per month is attached to the percentage mortality figures.

seasonal type, the greatest susceptibility to the toxin occurring during the first quarter of the year. This is followed by a marked fall in susceptibility between June and October. The perpendicular lines for each month represent twice the standard deviation, *i.e.* the range in which the true value of the mortality might be expected to lie with a probability of 0·95.

In titrating dysentery toxin and antitoxin by the League of Nations method (*vide* Sudmersen, Runge and O'Brien 1924, O'Brien and Runge 1925, Blake and Okell 1929), a variation of this type can be a serious source of error unless a control test of 1 test dose of toxin and a constant dose of standard serum giving a mortality ranging round 50% is made in parallel with each titration of an unknown serum. The value assigned to the unknown serum must be gauged by the comparative survival rates of the two groups of animals. It is hoped, however, by increasing the test dose of toxin as suggested in a recent paper (Blake and Okell 1929), that errors due to variations in susceptibility of mouse groups will be insignificant in ordinary titrations.

SUMMARY AND CONCLUSIONS.

In titrations of dysentery (Shiga) toxin and antitoxin, not only are variations in individual susceptibility to the toxin met with, but also variation effects in groups of mice used at intervals. The variability appears to be seasonal, the mean susceptibility of the mice to the toxin being greater in winter than in the summer months.

The possibility of variations of this type must be considered in titrations of any biological reagent where the degree of accuracy demanded approaches nearly to the extreme limits permitted by the method of test.

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TUMOUR-FORMATION FOLLOWING FREEZING WITH CARBON DIOXIDE SNOW.

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Received for publication March 5th, 1929.

SEVERAL workers have attempted to find a relationship between the vascular response brought about by a carcinogenic tar and the resulting tumour-formation. Itchikawa and Baum (1924) found that the skin of mice and rabbits, after tarring, had numerous dilated blood-vessels in the corium, while only a transient hyperæmia was seen in the tarred skin of guinea-pigs and rats. Since mice and rabbits readily developed tumours with tar whereas guinea-pigs and rats did not, they concluded that an adequate vascular reaction was an essential factor in the development of tumours. The finding of a relationship between the hyperæmia and the epithelial reaction after repeated tarring of the skin does not necessarily prove that the hyperæmia is a primary factor; it may only be secondary to the epithelial changes. Kotzareff (1926), and Rémond, Bernardbeig and Sendrail (1925) found that repeated tarring of the rabbit's ear brought on tumours at an earlier date if the superior cervical sympathetic ganglion of that side were excised previous to the experiment. Such an effect might be due to the resulting hyperæmia, though other changes

in the skin due to interference with the sympathetic nerve supply might be responsible. Auler, on the other hand, obtained entirely contrary results. The problem seemed of sufficient importance to warrant further investigation.

Many skin irritants are known to produce a local hyperæmia, and it was thought that skin subjected to such irritation might respond more readily to a carcinogenic tar than normal skin. But, as epithelium itself responds to mild injury by undergoing hyperplasia, it was considered advisable to undertake a preliminary investigation to discover whether repeated applications of such irritants alone would not be capable of inducing tumour growth.

Two forms of skin irritants were chosen—mild freezing with CO_2 snow, and applications of a dilute solution of mustard gas. The work in connection with the mustard gas has not yet been completed, and will appear as a separate communication at a later date.

EXPERIMENTAL PROCEDURE.

A pencil of CO_2 snow was applied to an area of skin on the backs of 70 white mice for about 3 seconds, and the experiment repeated at about weekly intervals. In order to avoid any unnecessary irritation of the skin no chemical depilator was used, but the hair was cut as short as was possible with a pair of curved scissors. The degree of freezing of the skin following an application of CO_2 snow depended largely on the amount of hair present, and in the early stages of the experiment it was found necessary to remove the hair previous to each application. This could be done with impunity by cutting the hair each time, whereas, had a chemical depilator been used, serious damage to the skin would have resulted. After a time the hair ceased to grow, and the bare patch of skin became thickened, with desquamation of fine epithelial flakes, leaving a smooth surface. Following an application of CO_2 snow, some reddening of the skin was soon visible, followed by a slight swelling resembling a small wheal. This was most noticeable in the early stages of the experiment. At a later stage the skin showed, microscopically, hyperplasia of the various layers of the epithelium, atrophy of the hair-follicles and sebaceous glands, and, in some cases, a mild inflammatory reaction in the corium. In 4 mice changes in the skin of quite a different order supervened:

Mouse 3M16.—After 6½ months a small raised nodule, which grew rapidly, appeared at the site of freezing, and in 6 weeks attained a diameter of ½ inch. It was removed under an anæsthetic and autoplasted in both groins. That in the right groin took successfully and grew to a large size, though an attempt to transplant it into other mice failed. Histologically the original growth had the appearance of a malignant tumour of spindle-cell type, with no evidence of cell-nest formation (fig. 1). A certain degree of continuity between the tumour and the hyperplastic epithelium at the margin, together with the microscopical appearance of the autoplast, suggested an epithelial origin. Such "spindle-cell carcinomata" are not infrequently met with in skin tumours of mice, the carcinomatous nature betraying itself by the presence of prickle-cells or keratin formation in part of the original tumour or in a secondary deposit or graft (*cf.* fig. 6). Involvement of the panniculus carnosus is seen in fig. 2, and a high-power view of the autoplast in fig. 3.

Mouse 2M2.—A small nodule appeared at the margin of the site of freezing

after 8½ months. The freezing was continued for a further 3 months, during which time the tumour grew slowly, and developed a thickened base and raised margin, with some ulceration of the surface. The animal developed an empyema and died. No secondary deposits were found. Histologically the tumour appeared similar to the previous one, with involvement of the panniculus carnosus (fig. 4).

Mouse 2M4.—A swelling developed at the area of freezing after 13 months, grew very slowly, and finally began to ulcerate. The animal died 2 months after the swelling was first noticed.

A small ulcerated wart was found, which, histologically, showed atypical growth but no invasion of muscle.

Mouse 1M5.—After 14 months' application of CO_2 snow a small ulcer appeared at the margin of the treated area: it became raised above the surface and grew rapidly; 17 days later the animal was found dead, apparently of hæmorrhage from the tumour.

Histologically the tumour was found to be a squamous carcinoma with typical cell-nest formation (fig. 5). The deeper part showed a spindle-cell appearance. In fig. 6 is seen a transition between carcinoma and sarcoma-like cells. Invasion of muscle is also seen.

The result, in short, is the production in 4 out of 70 mice of tumours of the skin by repeated freezing with CO_2 snow, 3 of them being malignant.

A similar investigation was undertaken on guinea-pigs, but an epidemic broke out among them, and the high mortality terminated the experiment prematurely. One observation is worthy of note: After several applications of CO_2 snow the hair ceased to grow in the area frozen, but at the periphery the hair grew more rapidly than in normal skin, so that a ring of long hair was seen with a bald patch inside and short hair outside. This may be of significance in the light of the experiments on mice described below. Other changes in the skin of guinea-pigs, following repeated freezing over a short period, need not be mentioned here, since they are fully described by Fuerst and others.

An experiment was then started to determine whether skin, subjected to repeated freezing with CO_2 snow, would be more prone than normal skin to develop tumours with tar.

A small patch of skin was frozen with CO_2 snow, and two or three hours later tar* was applied. Forty mice were used for the experiment proper, 40 mice received tar only (tar control), and 40 were treated with CO_2 snow alone (freezing control). This treatment was repeated about twice a week, and the experiment continued for 35 weeks. The freezing control, which was in reality a repetition of the first experiment, only yielded one doubtful result. This was probably due to the experiment not having been in progress for a sufficiently long period, and possibly to the fact that the freezing, though applied twice as often as in the first experiment, was milder in degree.

Mouse 25M2.—A small swelling appeared at the margin of freezing after 22 weeks. This remained small for 10 weeks and then disappeared. The animal is still alive.

* The tar used in these experiments was an ether extract of gas-works tar, treated with lime before the extraction.

A comparison of the results between the freezing plus tar series and the tar control is given in the accompanying table. The results show clearly

Table showing the Effect of Freezing on the Induction and Growth of Tar Tumours.

	Tar.	Freezing and tar.
Number of animals used	40	40
First appearance of a wart	9½ weeks.	9½ weeks.
First appearance of malignancy (judged clinically)	23 „	20 „
Number of animals bearing tumours at 20th week	10	12
Number of survivors at 20th week	24	31
Percentage of survivors bearing tumours at 20th week	41·7%	38·7%
Total number of animals bearing tumours*	20	26
Number which have become malignant†	14	9
Percentage of tumours become malignant	70%	34·6%

* The experiment was terminated at the 35th week, when all the survivors were killed for a microscopical study of the tumours.

† Involvement of muscle was taken as the criterion of malignancy.

that the development of tar tumours is not enhanced by the addition of repeated freezing of the skin. On the contrary, the figures would indicate that the freezing interfered with the rate of growth of the warts and hindered the tendency of the warts to become malignant, though no influence seemed to have been exerted on the original induction of these warts. Unfortunately the experiment was complicated by the fact that the tar which was applied over the frozen area of skin invariably spread beyond it, so that these mice had the central area subjected both to freezing and to tar, while a zone outside this received tar only. On no occasion did warts appear in the central area; a few developed well outside it, but the majority appeared at the immediate margin of the central area. It was only when the warts commenced to assume a malignant character that the centre showed invasion. These facts are well illustrated in fig. 7—a chart showing the development of warts of four mice treated with CO_2 snow and tar—while fig. 8 is a photograph of one of these mice, where a large number of warts are seen arranged in a semicircle at the margin of the frozen area. The growth of a ring of hair encircling an area of

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DESCRIPTIONS OF PLATES.

FIG. 1.—Mouse 3M16: Showing the marginal hyperplastic epithelium in the centre of the field and the tumour to the right. × 100.

FIG. 2.—Mouse 3M16: Another part of the same tumour showing invasion of muscle. × 120.

FIG. 3.—Mouse 3M16: Autoplast of the previous tumour. × 300.

FIG. 4.—Mouse 2M2: Tumour showing invasion of muscle. × 55.

FIG. 5.—Mouse 1M5: A squamous carcinoma with typical cell-nest formation. × 55.

FIG. 6.—Mouse 1M5: Another part of the tumour showing both squamous and spindle-cell formation. In the lower part of the field the tumour is invading muscle. × 120.

FIG. 8.—A photograph of Mouse 22M5, treated with CO_2 snow and tar. A number of warts is seen arranged round the central area of freezing.

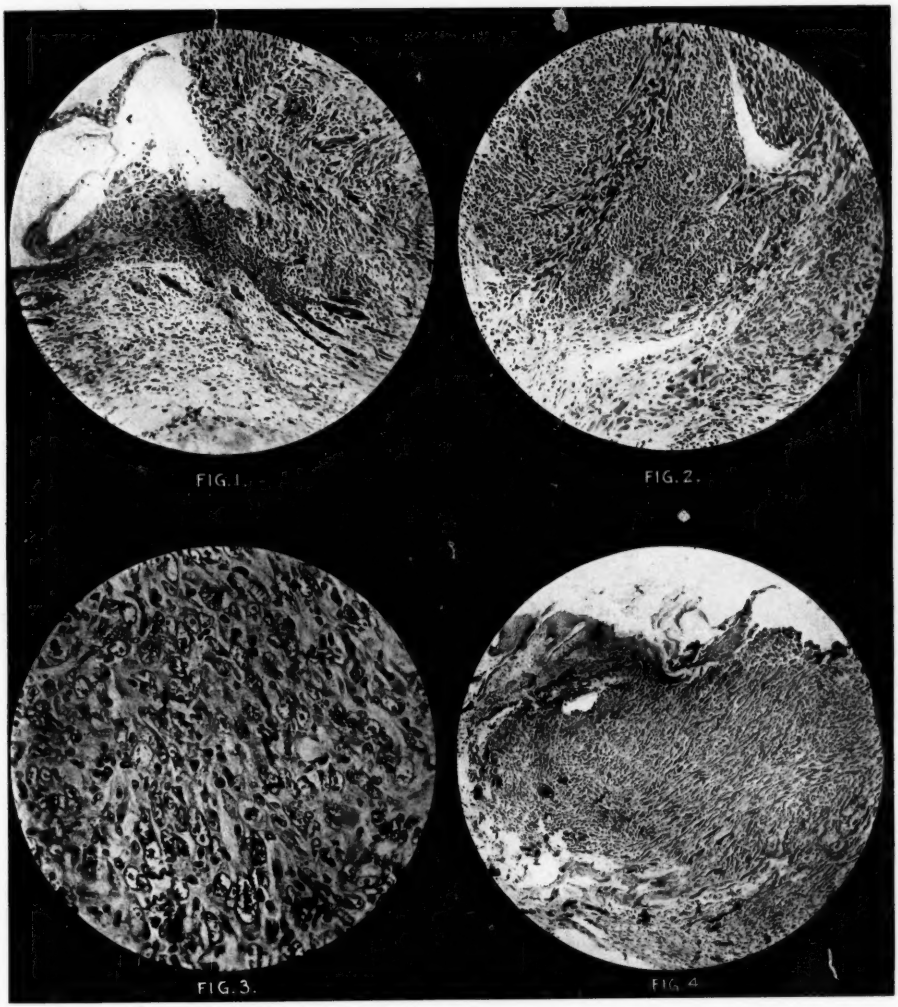

FIG. 1.

FIG. 2.

FIG. 3.

FIG. 4

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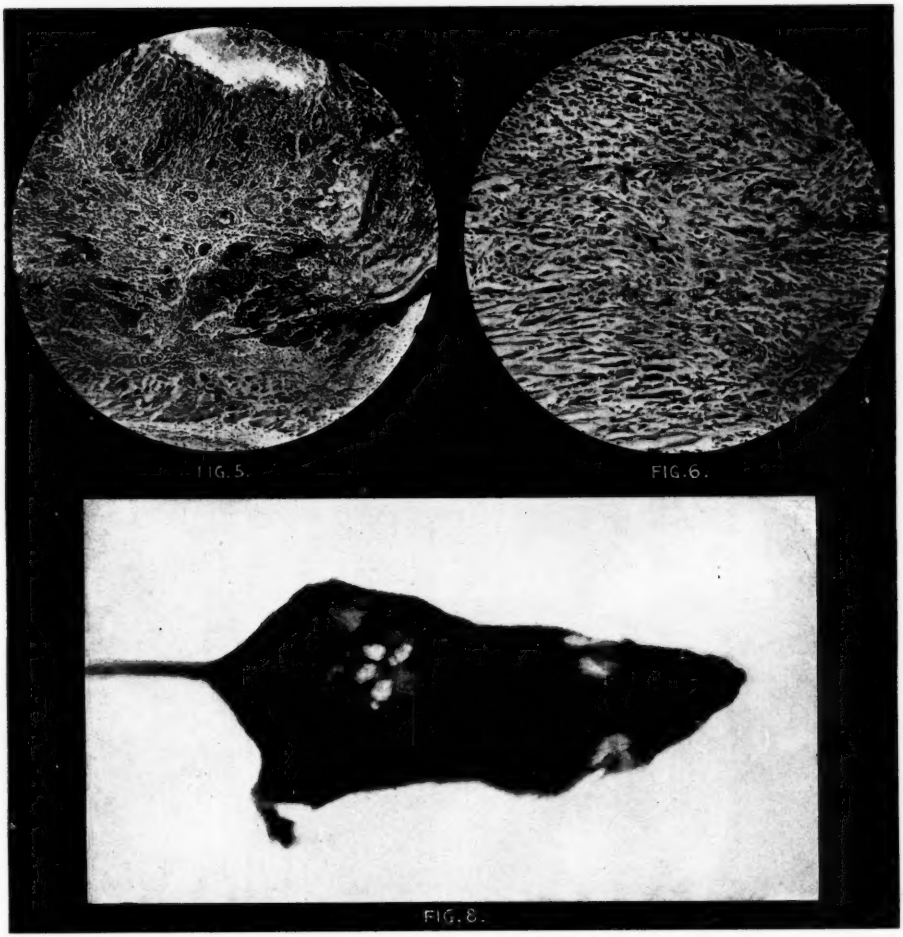

FIG. 5.

FIG. 6.

FIG. 8.

Berenblum.

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skin, frozen repeatedly with CO_2 snow, which was described above in connection with the experiment on guinea-pigs, is particularly interesting when taken in conjunction with the last-mentioned observation. A general comparison in the size of tumours after 35 weeks showed that in the tar control series they grew more rapidly and attained a larger size than in the freezing plus tar series.

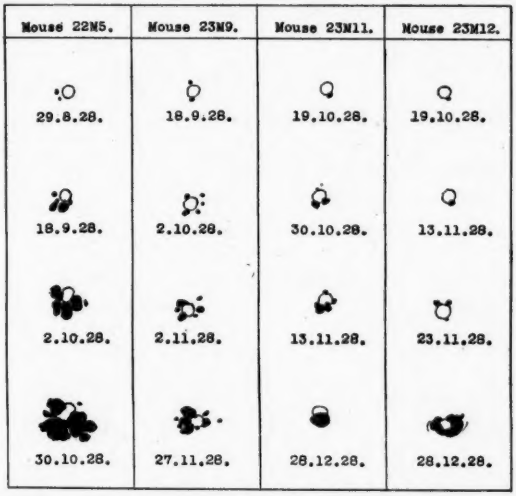

FIG. 7.—Drawings of warts at different stages in their development after repeated applications of CO_2 snow and tar. The rings outline the areas frozen, while the black masses represent the outlines of the warts. × 1½.

DISCUSSION.

The unexpected production of malignant tumours by repeated freezing with CO_2 snow is of interest clinically, since this agent is used for the treatment of various skin conditions. A very small proportion of the mice developed tumours, and the time necessary for their induction was 6–14 months. In comparison with an active carcinogenic tar (which is capable of producing tumours in practically 100% of mice, the earliest appearing in 9 weeks or less), repeated freezing with CO_2 snow cannot be considered as a very efficient carcinogenic agent. The duration of life of a man is at least 25 times that of a mouse, and it is generally assumed that the various processes occurring in a human body are at a correspondingly slow rate. Such calculations cannot be considered very accurate, but it is not altogether unreasonable to suppose that well over 12 years of repeated freezing would be needed to produce tumour-formation in man. Therefore it is not surprising that no tumours produced by the clinical use of CO_2 snow have been recorded in the literature.

The importance of the production of tumours in this way in mice lies in the fact that it is an example of experimental tumour-formation, where the

active external stimulus is a physical agent. Malignant new-growths following X-ray burns or developing in scars from ordinary burns are frequently recorded in clinical literature. Recently Findlay (1928) has been successful in producing tumours after repeated applications of ultra-violet light. The present results are possibly of the same order.

An explanation of the other results recorded is not easy: two carcinogenic agents, tar and CO_2 snow, when applied together, seem less capable of bringing about neoplastic changes in the skin than one of them (tar) alone. The work of Derom (1924) may be of interest in this connection. He found that the skin of mice, heated to 60° C. for a few seconds three times a week over a period of 4 months, was less liable to develop tumours by subsequent tarring than normal skin. (He was unable to produce tumours simply by heating the skin to 60° C. on alternate days for a period of 434 days. Bang (1925) has since shown that it was possible to produce tumours of the skin in mice after a single burn.)

A true antagonistic action between tar and freezing with CO_2 snow is unlikely. Perhaps there is an optimum degree of irritation which is most favourable for the ultimate change to tumour growth, so that an excess, whether by one irritant or by two, may be as unfavourable as an insufficient amount. Though such a supposition would be in keeping with the above results (which otherwise appear to be paradoxical), one would not be justified in formulating such a theory without further corroborative evidence.

SUMMARY.

1. Repeated mild freezing of the skin of mice with CO_2 snow over a long period may lead to the development of malignant tumours.

2. Only a small percentage of mice will respond in this manner, while the time necessary to produce such tumours is much longer than with the use of a carcinogenic tar.

3. When the skin of mice is subjected to repeated freezing and tarring, warts appear at the periphery of the area frozen in about the same period of time as with tar alone. No warts appear to develop in the frozen area itself.

4. The warts produced in mice treated with CO_2 snow and tar do not grow as rapidly, nor become malignant as soon, as those produced by tar alone.

In conclusion I should like to express my indebtedness to Prof. R. D. Passey for suggesting these experiments, and for help and advice generously given during the course of this work.

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INNERVATION AND TUMOUR GROWTH.

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Received for publication March 10th, 1929.

WHEN carrying out experimental work in 1926 in the Histological Laboratory of the University of Leyden on the problem of innervation and cancer, the writer made the following observations:

Fifty mice were painted with tar on the skin of the back in the usual way well known to investigators engaged on cancer research. Every other day an animal was killed and a piece of the treated skin taken out and stained, with a view to studying the reactions, if any, of the nerves of the irritated area; a well-staining Cajal impregnation gave excellent results.

It was found that even after 2 weeks the nerves begin to grow out of their normal fascicles; very thin nerve-threads, mostly non-medullated, grow towards the epithelium, giving a curious irregular appearance to the silver preparations.

This phenomenon continues, and gradually becomes intensified to such an extent that it can be described as *hyperneuria*. This is observable after 6 weeks of tar application, and thereafter continues, although accompanied by an opposite phenomenon, viz. a decrease of the normal tendency of the nerves to establish close contact with the cells of the epithelium; whilst their number increases, their tendency to adhere to the epithelium is not above normal.

It is even noticeable that those threads which are originally directed towards the surface are driven back and tend to the opposite direction; this peculiarity was called *katatropism*. The first type of reaction is, however, maintained, and hyperneuria gains in volume to the extent of more than one hundred times the normal innervation—a truly remarkable microscopic observation. Simultaneously, the negative katatropism increases and small areas of epithelium are denervated. The malignant new-growth is thus entirely devoid of nerves.

It was further demonstrated that hyperneuria is not a reaction specific to the application of tar; mechanical lesions, burning and certain other peripheral stimulations also produced hyperneuria, although of a much less pronounced character. Even the mere regeneration after the dissection of a nerve-trunk produces some superfluous fibres, which in essence does not differ from the reaction described above, being no more than a special instance of the hyperneuria as caused by any stimulation.

The above observations had been published when W. Cramer's paper, published in this Journal in April, 1925, came to my notice and suggested an interesting connection between his experiments and the writer's observations; indeed, they seemed to fill a gap in the problem of innervation and tumour growth.

W. Cramer introduced a most ingenious technique: An oblong piece of skin, about 2 cm. long and 1½ cm. wide, was cut out and sewn in again, so that it was freed from any nervous control. After two weeks, if the graft was successful, tar-painting was commenced on the autoplast as well as on the normal skin, and continued for more than 24 weeks. His conclusions are given in the following words: "Loss of nervous control in an area of skin does not predispose to cancer. On the contrary the presence of a peripheral nervous mechanism is an essential factor in the process of chronic irritation which leads to the development of cancer."

It was at once recognized as a remarkable fact that, according to this investigation, regeneration in the autoplast did not take place until three months, or longer, after the operation.

As the writer's observations seemed to indicate that mere tar-painting produces rapid and abundant innervation, as described above, in less than two months, the discrepancy appeared worth investigating.

With this object in view Cramer's "autoplast" technique was adopted, but not the "flap" method as described in his paper, in order to make sure that no nerves could escape the scissors.

Several animals were operated upon for this purpose, and a series of successful grafts were stained with the Cajal impregnation at periods from 2 to 49 days after the re-implantation. No tar-painting was, of course, applied in the experiments.

It was found that degeneration of all nerves still visible after the first day had set in on the second day; microscopic observation confirmed what would be expected *à priori*.

On the second day there was on the other hand clear evidence of regeneration of the nerve-trunks cut in the circumferential cross-section of the skin (see Fig. 1); after the exhaustive treatment of this subject by Cajal (1928) and many others, it is unnecessary to refer to these observations otherwise than by describing them as "typical."

After 5 days the autoplast is completely free from nerves, but the border of normal tissue is in full process of regeneration.

After 18 days (no intermediate stages having, unfortunately, been studied) the situation can be analysed as follows:

The process of healing, as observed in preparations made by ordinary histological methods, is nearly complete. The absence of muscular fibres and the distorted form of the hair-roots indicate the place of the autoplast. Nerve-fibres have grown in, and at several places, even at more than 0·3 cm. from the border, the fine threads with their "cones de croissance" penetrating to the corium-tissue mostly in a horizontal direction are clearly visible. Several of these touch the epithelium, have reached old or new hair-roots, and the rest have their bud-shaped terminations penetrating into the connective tissue. Most of them escape from the surrounding skin, as might be expected. No fibres are found growing up into the central portions of the autoplast (see Fig. 2). There is still a small centre in which no nerve-fibres are to be seen at all.

After from 24 to 30 days such a centre could not be found in the series of sections prepared of the grafted skin; at that time nerves are visible

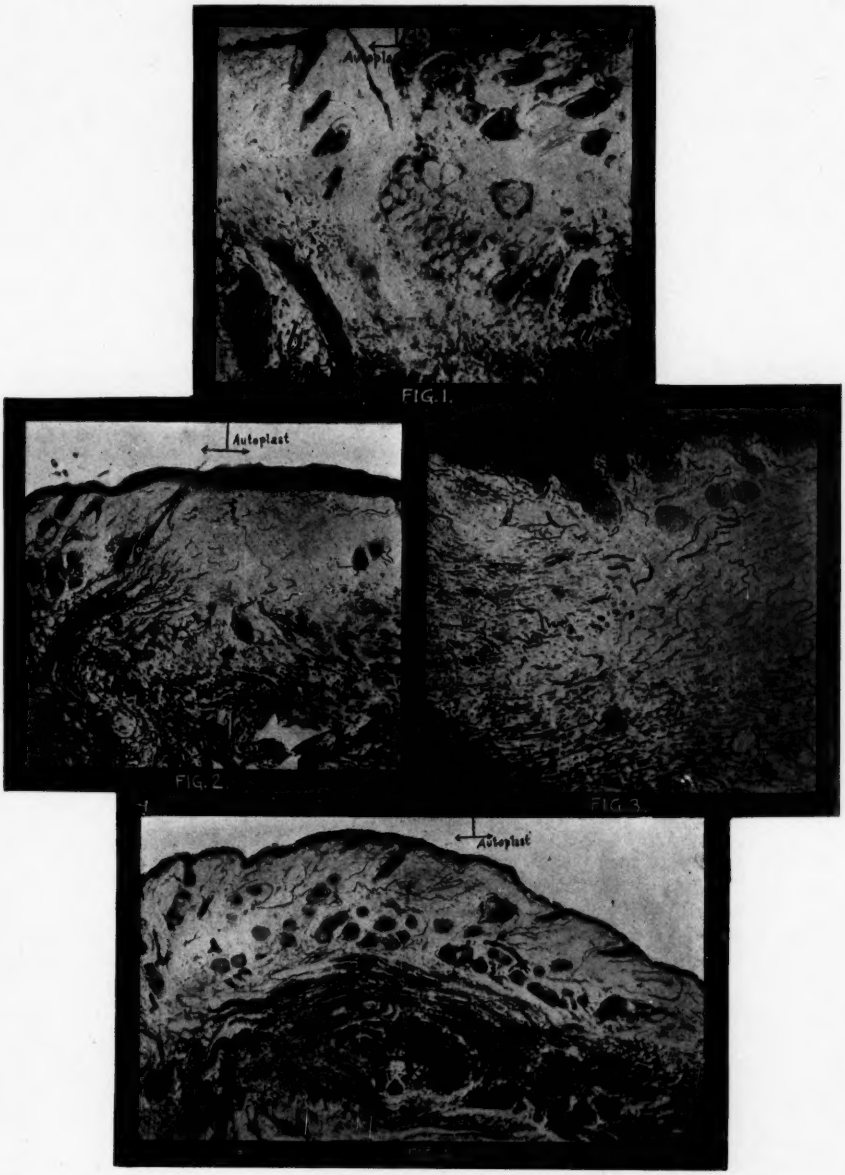

FIG. 1.—Two days after operation: (*a*) Nerve-trunk in the normal skin, showing regeneration in an early stage; (*b*) nerve-trunk in the autoplast, no more than a few fibres being stained.

FIG. 2.—Eighteen days after operation. Nerve-fibres with their "cônes de croissance" penetrating into the autoplast.

FIG. 3.—A place in autoplast tissue, 29 days after operation.

FIG. 4.—Thirty-three days after operation; beginning of the hyperneuria.

Julius.

everywhere in the autoplast, as shown in Fig. 3, which it is unnecessary to describe in detail. Young hair-roots, with active, young innervation, show that the tendency to a regeneration *ad integrum* is present.

The only point remaining to describe is the innervation-image of an autoplast 41 days old. The nerve supply now is richer than in the normal skin (see Fig. 4); this phenomenon had also been observed in the writer's earlier experiments, and in all cases of nerve out-growth after any kind of irritation (see Boeke, 1916). The hair-roots have too many nerve-endings, but show a tendency to normal shape (ring and palisade), whilst everywhere in the connective tissue and epithelium a large number of threads are found.

It was thus established that already after 18 days a denervated area, such as it was intended to produce by the autoplast operation, did not exist, whilst after 6 weeks the effect of the intervention was in the direct opposite direction. Comparing these observations with the known nerve-reactions after tar-painting only, it seems admissible to conclude that Cramer in his above-mentioned researches applied tar to denervated areas during 4 to 8 days only; after that period he painted on skin with normal nervous mechanism, and still later, after a few weeks, on skin with hyperneuria of a double origin.

Further research will be necessary before definite inferences can be drawn from these facts; but already now it seems evident that while Cramer's conclusions may, of course, be verified by subsequent observations, they cannot be logically derived from his experiments under discussion.

SUMMARY.

When an oblong piece of skin $2 \times 1\frac{1}{2}$ cm. is cut out of the back of a mouse and sewn on again, with a view to obtaining an autoplast free from nerves, regeneration of the nerves begins on the second day. Nerve-threads are present in the autoplast in less than 18 days, and complete innervation is re-established within 25 days.

Hyperneuria sets in after 40 days.

The above, combined with the fact that tar-painting on the normal skin of mice causes excessive hyperneuria, indicates that W. Cramer, when carrying out the experiments described in this Journal (1925, **6**, 71), did not apply the tar on a denervated skin area, and that therefore his deductions may need revision.

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VIRUS III. IN TISSUE CULTURES. I.—THE APPEARANCE OF INTRANUCLEAR INCLUSIONS *IN VITRO*.

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Received for publication March 21st, 1929.

A NUMBER of viruses have now been shown to multiply in tissue cultures. It is also well known that in many virus infections remarkable changes are found in the cytoplasm or nucleus of affected cells (inclusion bodies); these inclusions may be specific, as in rabies or fowl-pox, or very similar changes may be produced by infections with certain closely allied viruses such as varicella, herpes and virus III of rabbits. Yet the appearance of these inclusions in tissue cultures has not been described,* apart from a paper by Gins in 1916.

Virus III (Rivers and Tillett, 1923) is the name given to a virus endemic amongst certain stocks of laboratory rabbits. Infective material injected into the testis of a rabbit produces acute orchitis; intradermal inoculations lead to a definite erythematous lesion within 3 or 4 days. Histologically many cells in the interstitial tissues of the testis and elsewhere are found to contain large acidophilic inclusions within the nucleus, separated from the basophilic nuclear membrane by a clear zone (Rivers and Tillett, 1924).

During the last few months I have found that virus III readily multiplies in tissue-cultures of adult rabbit testis, and that the intranuclear inclusions are regularly formed in these cultures. It will be apparent from the following description of the technique that the "tissue-culture" is of a simple and primitive kind, and that the term "cell-survival" might be more appropriate.

TECHNIQUE.

A normal rabbit's testis was minced aseptically as finely as possible with scissors, and small pieces—0·05 to 0·1 gm. in all—were spread with a platinum spatula on the bottom of a 2-in. Carrel D flask (Carrel, 1923). 0·1 c.c. of an emulsion of infected testis in Ringer's solution or 0·1 c.c. of the fluid portion of another culture was dropped in, and immediately afterwards 2 c.c. of rabbit plasma diluted 1 in 4 (in a few experiments 1 in 5) with Tyrode's solution were added. The plasma was prevented from clotting prematurely by the use of heparin in a final concentration of 1 in 1000; in presence of the tissue clotting almost always occurred satisfactorily within a few minutes or at most a few hours. No tissue or embryonic extract was used. The necks of flasks were closed with cotton-wool plugs and rubber caps and incubation was

* Within the past few days I have heard by letter from Dr. T. M. Rivers of the Rockefeller Institute, New York, that he, with Drs. E. Haagen and R. S. Muckenfuss, has recently noted the appearance of the characteristic inclusions of vaccinia and of herpes simplex when these viruses are grown in tissue cultures of rabbit cornea. An account of these observations is to appear shortly in the *Proc. Soc. Exp. Biol. Med.*

carried out at 37° C. Cells could be seen migrating from the edges of the fragments of testis within a day or two. After 3, 4 or 5 days of incubation the cultures were tested for the presence of virus in one or more of three ways: (*a*) the fluid portion, undiluted or diluted 1 in 10 or 1 in 100, was inoculated intradermally into the epilated skin of a rabbit; (*b*) the undiluted fluid was injected into a rabbit's testis, which was examined histologically after 4 or 5 days; (*c*) the fragments of testis within their clot were fixed in Bouin's solution, and sections, stained with hæmatoxylin and eosin, examined for intranuclear inclusions.

In some experiments the technique was modified in that filtered rabbit serum (similarly diluted with Tyrode's solution) was used instead of plasma. This technique is a simplification of that described by H. B. and M. C. Maitland (1928) for the cultivation of vaccinia virus.

RESULTS.

The table shows that virus III was readily carried in this way through ten successive cultures with hardly any failures. Since the virus was diluted more than 20 times at each transfer and its initial titre was 1 in 100, this

TABLE I.—*Results of Tests for Presence of Virus in 16 Cultures of Virus III; of these, 10 are in consecutive series.*

Culture.	Material used to infect culture.	Medium.	Number of flasks inoculated.	Number of flasks positive.	Criterion of presence of virus.		
					Skin "take."	Testis "take."	Inclusions in culture material.
1A	Fresh testis emulsion	Plasma	3	3	+	...	+
1B	„ „	Serum	3	2	+	...	...
2A	Fluid from culture 1A	Plasma	2	2	+	+	+
2B	„ „ 1B	Serum	2	2	+	...	...
3	„ „ 2A	Plasma	2	2	+	+	...
4A	„ „ 3	„	2	2	+	+	+
4B	„ „ 3	Serum	2	2	+	+	?
5	„ „ 4A	Plasma	2	2	+	+	+
6A	„ „ 5	„	2	2	+	+	+
6B	„ „ 5	Serum	2	1	+	...	?
7A	„ „ 6B	Plasma	2	2	+	...	+
7B	„ „ 6B	Serum	2	2	+	...	+
8	„ „ 7B	„	2	2	+	...	+
9	„ „ 8	„	2	2	+	...	+
10A	„ „ 9	Plasma	2	2	+	...	+
10B	„ „ 9	Serum	2	2	+	...	+

+ = positive reaction skin or testis or presence of inclusions in fixed tissue; ... = no test made.

represents a multiplication of at least $\times 10^{11}$. It will be noted that the last 5 cultures in the series were in flasks in which serum was used; thus in the serum medium a multiplication of at least $3{\cdot}2 \times 10^6$ had taken place. These figures are more striking if it is remembered that the virus always died out in 4 days if it was incubated under similar conditions with plasma alone (no tissue) or with tissue which had been boiled for 5 minutes, or where testis of the guinea-pig (a species not susceptible to virus III) was substituted for rabbit testis. It will be seen that where material from one culture was used to inoculate another the virus grew successfully in 27 out of 28 flasks.

Where fresh testicular emulsion was used to inoculate the cultures, the proportion of successes was almost as high (12 out of 14 flasks) whether serum or plasma was used.

HISTOLOGICAL EXAMINATION OF CULTURES OF NORMAL TESTES, INFECTED *IN VITRO*.

The larger fragments of testis in plasma always showed necrosis of tissue centrally, but fairly healthy-looking cells at the periphery. Here numerous typical inclusions were regularly found; they were not seen in the cells at the extreme edge which were actually migrating into the clot, but in cells in the interstitial tissues, being in fact in the same position as would be expected in a testis infected *in vivo*. Of 11 plasma-cultures fixed and examined, inclusions were numerous in 10; they were absent from the eleventh, but in this culture alone was the virus shown by skin tests to have died out.

Fourteen pieces of tissue from serum-cultures have also been fixed and studied; in 13 of these changes have been seen which seem to represent the early stages of inclusion-formation—in some cells the nuclear membrane is basophilic, while the rest of the nucleus is slightly acidophilic and seems to be shrinking away from the nuclear membrane; in 11 sections fully-formed inclusions were seen. It seems likely that in the serum medium the cells may live not quite or only just long enough to allow the full cycle of changes to occur; but it is almost certain that multiplication of the virus is associated with some survival of the cells for at least a day or two.

CONTROL EXPERIMENTS.

Flasks prepared and incubated as described but not seeded with virus invariably failed to give a skin reaction (14 flasks tested). Material from such control flasks was "subcultured" side by side with the infective material for five successive transfers, and no evidence of virus growth ever appeared. Nothing resembling an intranuclear inclusion was seen in tissue from any of the control flasks.

This is a preliminary communication. It is hoped to publish later the results of work now in progress on the application of the technique described to the study of immunological and other problems in virus infections.

SUMMARY.

(1) Virus III multiplies in adult rabbit testis which is surviving in "tissue-cultures" in dilute rabbit plasma or serum. It has been carried through 10 serial cultures, corresponding to a multiplication of over 10^{11} times.

(2) The intranuclear "inclusions" associated with infection with this virus are regularly formed in the plasma-cultures; they may also be found in serum-cultures.

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THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY.

VOL. X, No. 2. APRIL, 1929.

CONTENTS.

BURNET, F. M.—A METHOD FOR THE STUDY OF BACTERIOPHAGE MULTIPLICATION IN BROTH 109

KELLAWAY, C. H.—ANAPHYLACTIC STUDIES WITH EXTRACTS OF HYDATID SCOLICES 115

ELFORD, WILLIAM JOSEPH.—ULTRA-FILTRATION METHODS AND THEIR APPLICATION IN BACTERIOLOGICAL AND PATHOLOGICAL STUDIES . . 126

BLAND, J. O. W.—FILTER AND CENTRIFUGE EXPERIMENTS WITH NEUROVACCINE 145

FILDES, PAUL.—TETANUS.—VIII. THE POSITIVE LIMIT OF OXIDATION-REDUCTION POTENTIAL REQUIRED FOR THE GERMINATION OF SPORES OF *B. TETANI IN VITRO* 151

BLAKE, ADELAIDE V., AND OKELL, C. C.—SEASONAL VARIATION IN THE SUSCEPTIBILITY OF MICE TO DYSENTERY (SHIGA) TOXIN 175

BERENBLUM, I.—TUMOUR-FORMATION FOLLOWING FREEZING WITH CARBON DIOXIDE SNOW 179

JULIUS, H. W.—INNERVATION AND TUMOUR GOWTH 185

ANDREWES, C. H.—VIRUS III. IN TISSUE CULTURES. I.—THE APPEARANCE OF INTRANUCLEAR INCLUSIONS *IN VITRO* 188

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